Analytical Profiles of Drug Substances

Volume 19

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ACADEMIC PRESS, INC.
Harcourt Brace Jovanovich, Publishers
San Diego New York Boston London Sydney Tokyo Toronto

Academic Press Rapid Manuscript Reproduction

This book is printed on acid-free paper. ♾

ACADEMIC PRESS, INC.
San Diego, California 92101

United Kingdom Edition published by
ACADEMIC PRESS LIMITED
24-28 Oval Road, London NW1 7DX

LIBRARY OF CONGRESS CATALOG CARD NUMBER: 89-659072

International Standard Serial Number: 0099-5428

ISBN 0-12-260819-4 (alk. paper)

PRINTED IN THE UNITED STATES OF AMERICA
90 91 92 93 10 9 8 7 6 5 4 3 2 1

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PREFACE

Although the official compendia define a drug substance as to identity, purity, strength, and quality, they normally do not provide other physical or chemical data, nor do they list methods of synthesis or pathways of physical or biological degradation and metabolism. Such information is scattered through the scientific literature and the files of pharmaceutical laboratories.

I perceived a need to supplement the official compendial standards of drug substances with a comprehensive review of such information, and eighteen years ago the first volume of *Analytical Profiles of Drug Substances* was published. That we have been able to publish one volume per year is a tribute to the diligence of the editors to solicit articles and even more so to the enthusiastic response of our authors, an international group associated with pharmaceutical firms, academic institutions, and compendial authorities. I would like to express my sincere gratitude to them for making this venture possible.

Over the years, we have had queries concerning our publication policy. Our goal is to cover all drug substances of medial value, and therefore, we have welcomed any articles of interest to an individual contributor. We also have endeavored to solicit profiles of the most useful and used medicines, but many in this category still need to be profiled.

Klaus Florey

ACEBUTOLOL

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T6G 2N8

1. DESCRIPTION

1.1 Nomenclature

1.1.1 Chemical Names

N-[3-Acetyl-4-[2-hydroxy-3-[(1-methylethyl)amino]-propoxy]phenyl]butanamide (1,2); 3′-acetyl-4′-[2-hydroxy-3-(isopropylamino)propoxy]butyr-anilide; 1-(2-acetyl-4-n-butyramidophenoxy)-2-hydroxy-3-isopropylaminopropane; 5′-butyramido-2′-(2-hydroxy-3-isopropylaminopropoxy)-acetophenone. IL-17803A, M & B 17803A (1). Chemical abstracts registry no.: 37517-30-9; 34381-68-5 (hydrochloride).

1.1.2 Nonproprietary Name

Acebutolol (1)

1.1.3 Proprietary Name

Acetanol, Acecor, Diasectral, Neptall, Monitan, Prent, Sectral, Secradex (1,2).

1.2 Formula

1.2.1 Empirical

$C_{18}H_{28}N_2O_4$; Hydrochloride, $C_{18}H_{29}ClN_2O_4$

1.2.2 Structural

Figure 1 depicts the structure of acebutolol and its major metabolites, acetolol and diacetol.

1.3 Molecular Weight

336.43 (base); 372.89 (hydrochloride)

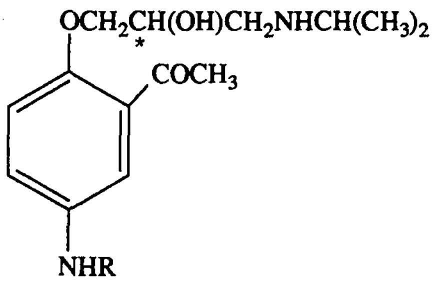


FIGURE 1. Structure of acebutolol and two metabolites, acetolol and diacetolol. Acebutolol, R = $CO(CH_2)_2CH_3$; Diacetolol, R = $COCH_3$; Acetolol, R = H. The asterisk (*) denotes the chiral center.

1.4 Appearance, Color and Odor

A fine white, or slightly off-white crystalline, non-hygroscopic, practically odorless powder (3).

2. SYNTHESIS

The synthesis of acebutolol has been described as follows (2):

After preparation of 5′-butyramido-2′-(2,3-epoxypropoxy)acetophenone, the compound was heated together with isopropylamine and ethanol and allowed to reflux for 4 hours (for details on preparation of 5′-butyramido-2′-(2,3-epoxypropoxy)acetophenone, see ref. 2). The reaction mixture was concentrated and dissolved in hydrochloric acid. This solution was extracted with ethyl acetate and the organic layer was discarded. Using 2 N sodium hydroxide, the acidic solution was basified and extracted with chloroform. The chloroform extracts were concentrated, yielding an oil. The oil was crystallized from a mixture of

ethanol and diethyl ether to give the final product, 5'-butyramido-2'-(2-hydroxy-3-isopropylaminopropoxy)-acetophenone (acebutolol).

A general scheme depicting the synthesis of acebutolol has also been reported, and is shown in Figure 2 (4).

3. PHYSICAL PROPERTIES

3.1 Infrared Spectra

The infrared spectrum of acebutolol is presented in Figure 3. The spectrum was obtained from a KBr disk using a Nicolet 7199 Fourier Transform infrared spectrometer. Diagnostic peaks were observed at 3420 cm^{-1} (alcohol, O-H stretch, H-bonded); 3350 cm^{-1} (secondary amine, N-H stretch); 3000-2850 cm^{-1} (alkane, C-H stretch); 1700 cm^{-1} (ketone, C=O stretch); and 1660 cm^{-1} (amide, C=O stretch). The peaks are presented in Table I. The infrared spectrum of acebutolol HCl has previously been reported which identified peaks at 1665, 1245, 1525, 1495, 1217 and 1285 cm^{-1} (5).

3.2 NMR Spectra

The 300 MHz proton NMR spectrum of acebutolol in $CDCl_3$ is described in Table II. The spectrum was obtained on a Bruker AM-300 FT NMR spectrometer. Instrumental settings were: time domain (data points), 16K; aquisition time, 1.819 sec.; spectral width, 4504.51; pulse width, 25°; receiver gain, 200; line broadening, 0.200. The spectrum is shown in Figure 4.

The D_2O exchange NMR spectrum of acebutolol is shown in Figure 5. As expected, the exchangeable protons (-OH, -NH-, -CONH) are absent, whereas a single, intermediate peak is present at 4.83δ.

3.3 Mass Spectra

A mass spectrum was obtained on a AEI MS9 (Manchester, U.K.) instrument equipped with a fast atom bombardment source (Figure 6). The medium was glycerol and the sample was introduced by means of a direct insertion probe. Instrument settings were: mass range, 93-676; total scans in run, 4; sampling rate, 128; signal level threshold, 30; minimum peak width, 7; scan rate (sec/dec), 10.0.

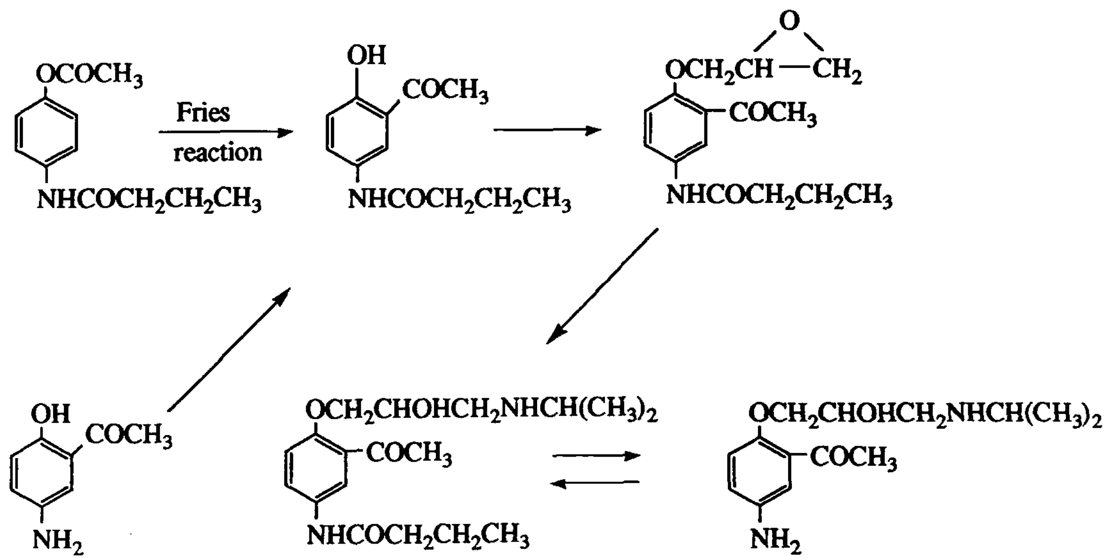


FIGURE 2. Synthetic Pathway for Acebutolol (from ref. 4).

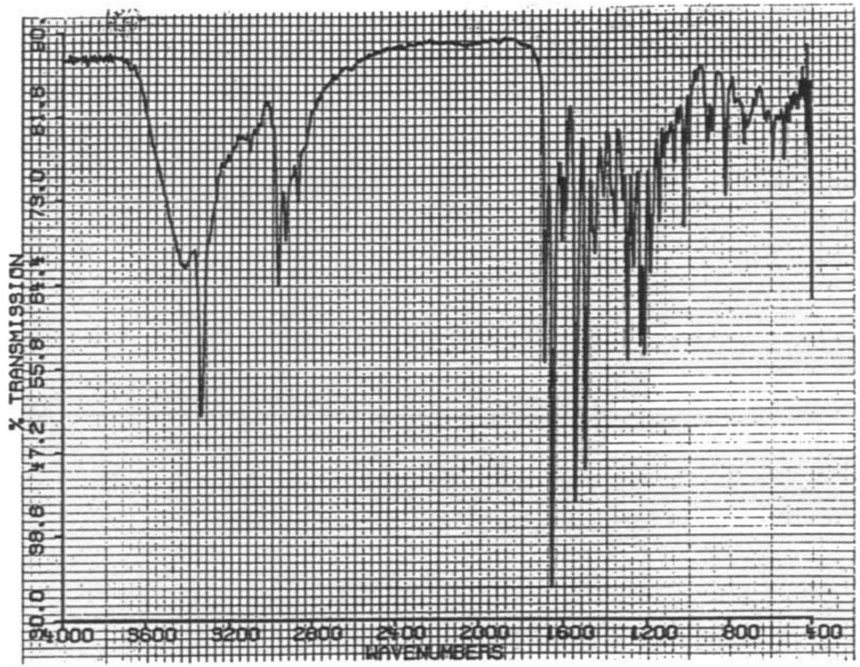


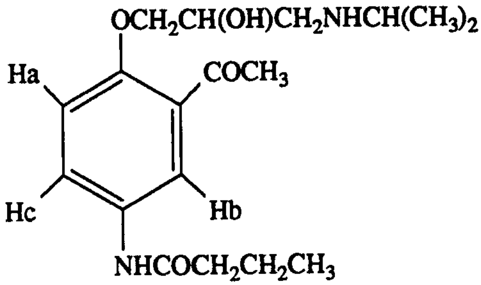
FIGURE 3. Infrared spectrum of Acebutolol. KBr pellet.
Instrument: Nicolet 7199 Fourier Transform Infrared Spectrometer.

TABLE I. I.R. Spectrum of Acebutolol. KBr pellet.
(Instrument: Nicolet 7199 Fourier Transform I.R.)

Wavenumber (cm^{-1})	Relative Intensity[a]
3424	m, broad
3385	s
2965	m
2931	w
2880	w
1692	s
1654	s
1612	w
1593	w
1545	s
1498	s
1467	w
1410	w
1358	w
1299	s
1270	m
1237	s
1219	s
1189	m
1146	w
1024	w
910	w
880	w
820	w
590	w
538	w

[a] s = strong; m = medium; w = weak.

TABLE II. 300 MHz Proton NMR of Acebutolol in $CDCl_3$.



Chemical Shift (rel. to TMS)	Number of Protons	Assignment
1.02 t	3	$C\underline{H}_3CH_2$-
1.11 d	6	$-CH(C\underline{H}_3)_2$
1.76 sextet	2	$CH_3C\underline{H}_2CH_2$
2.05 broad	2	$-N\underline{H}-$, $-O\underline{H}$
2.35 t	2	$-CH_2C\underline{H}_2CONH$
2.67 singlet	3	$-COC\underline{H}_3$
2.8 m	3	$-C\underline{H}_2NH-$, $-C\underline{H}(CH_3)_2$
4.08	3	$-OC\underline{H}_2-$, $-C\underline{H}OH-$
6.96 d	1	-Ha
7.34 broad, singlet	1	$-CON\underline{H}$
7.63 d	1	-Hb
7.95 q	1	-Hc

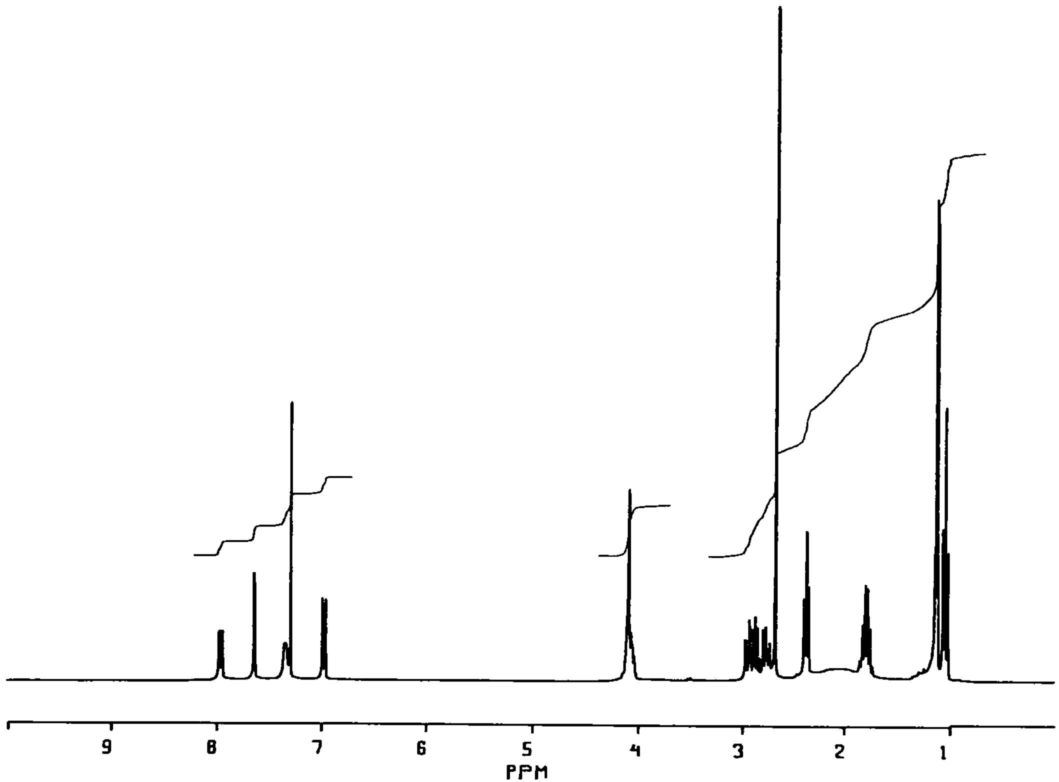


FIGURE 4. Proton NMR Spectrum of Acebutolol.
Instrument: Bruker AM-300 FT NMR spectrometer

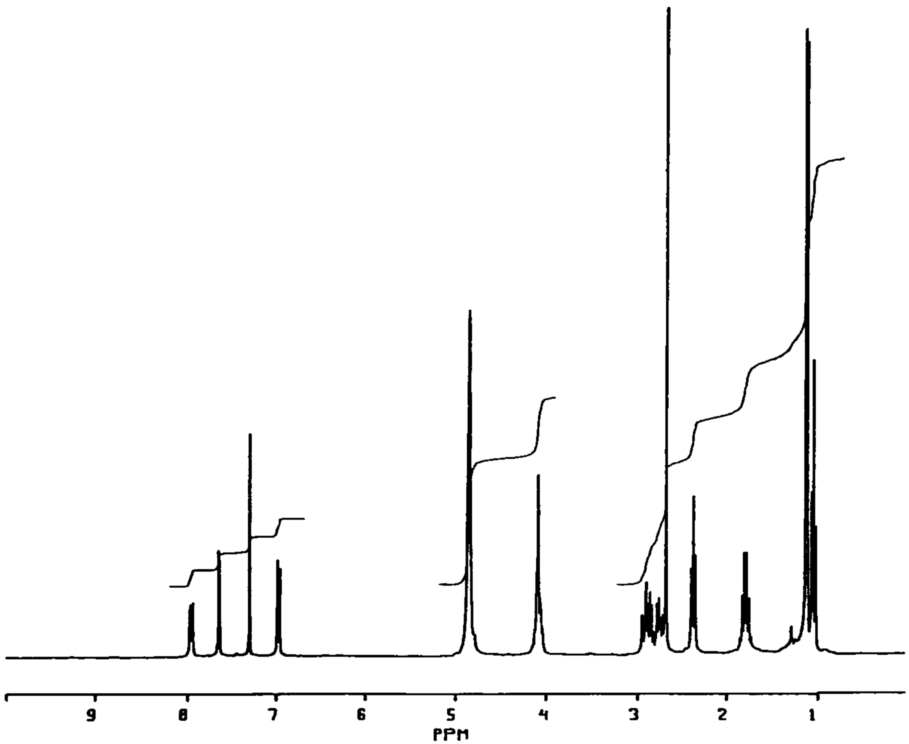


FIGURE 5. Proton NMR Spectrum of Acebutolol. D_2O exchange.
Instrument: Bruker AM-300 FT NMR spectrometer.

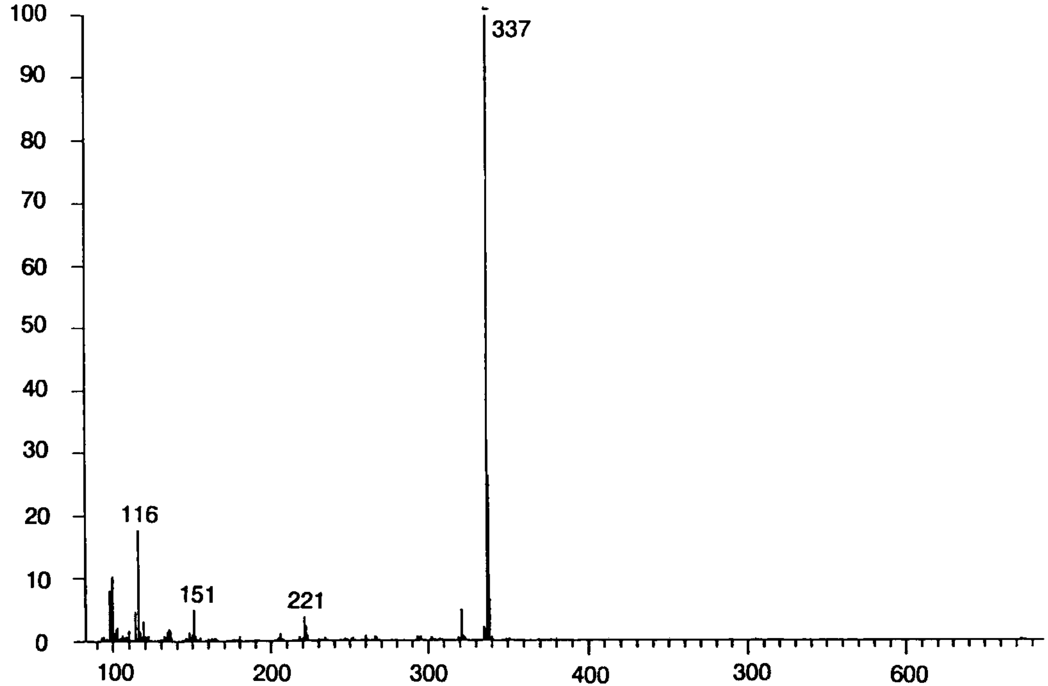


FIGURE 6. Positive Ion FAB Mass Spectrum of Acebutolol.
Instrument: AEI, MS9. Medium, glycerol.

Acebutolol gave a prominent peak (base peak) [MH^+] of m/z 337 in the positive ion detection mode. Fragments were detected at m/z (% relative abundance): 98(8), 100(10), 116(19), 151(5), 338(26) and 339(6). The fragmentation pattern for the positive ion mode has been presented in Figure 7.

A previously reported mass spectrum identified peaks at m/z 72, 43, 30, 56, 151, 221, 41 and 98 (5), although the mass spectrometry conditions were not listed.

Using electron-impact mass spectrometry, with ionization energy of 70 eV (Hewlett-Packard MSD Series 5970 A), acebutolol reportedly gave prominent fragments of m/z 108, 136, 151, 193 and 235. In this previous report, the authors concluded that acebutolol had been hydrolysed to an aminophenol derivative under the stated conditions (6).

3.4 Ultraviolet Spectrum

The ultraviolet spectrum of acebutolol in chloroform obtained using a PU8700 series UV/VIS spectrophotometer (Philips, England) is depicted in Figure 8. This qualitative spectrum depicts maximal wavelengths at 241.6 and 328.5 nm under the stated conditions. The spectra, as reported by others (5), were: 1) aqueous acid, 234 nm (A^1_1 = 655, unknown reliability) and 320 nm (A^1_1 = 75, unknown reliability), and in 2) methanol, 235 nm (A^1_1 = 866, mean value based on several reported figures, all within ±10% of the mean), and 328 nm.

3.5 Melting Point

Acebutolol melts within the range of 119-123 °C (1, 5). The hydrochloride salt has a melting point of 141-144 °C (5).

3.6 Dissociation Constant

The pka of acebutolol is 9.4 (5).

3.7 Partition Coefficient

The partition coefficients of acebutolol in n-octanol/phosphate buffer, incubated for 1 hour, were: 0.17 (20 °C, pH 7.0); 0.35 (37 °C, pH 7.0); and 0.68

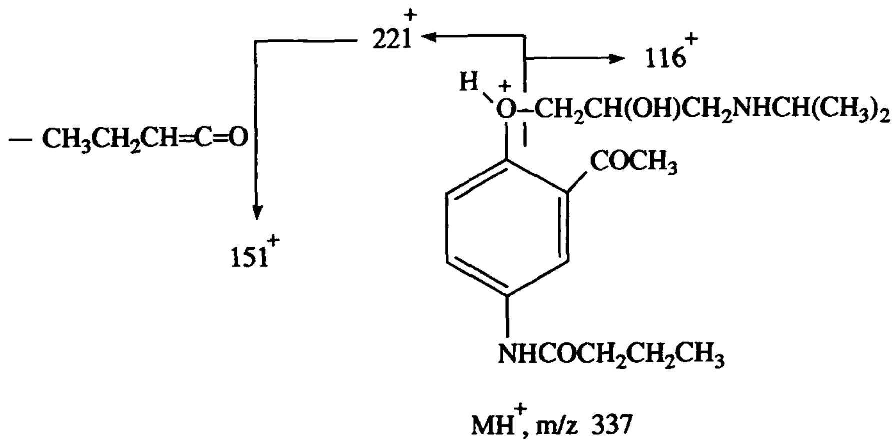


FIGURE 7. Fragmentation Pattern of Acebutolol; Positive Ion FAB Mass Spectrum.

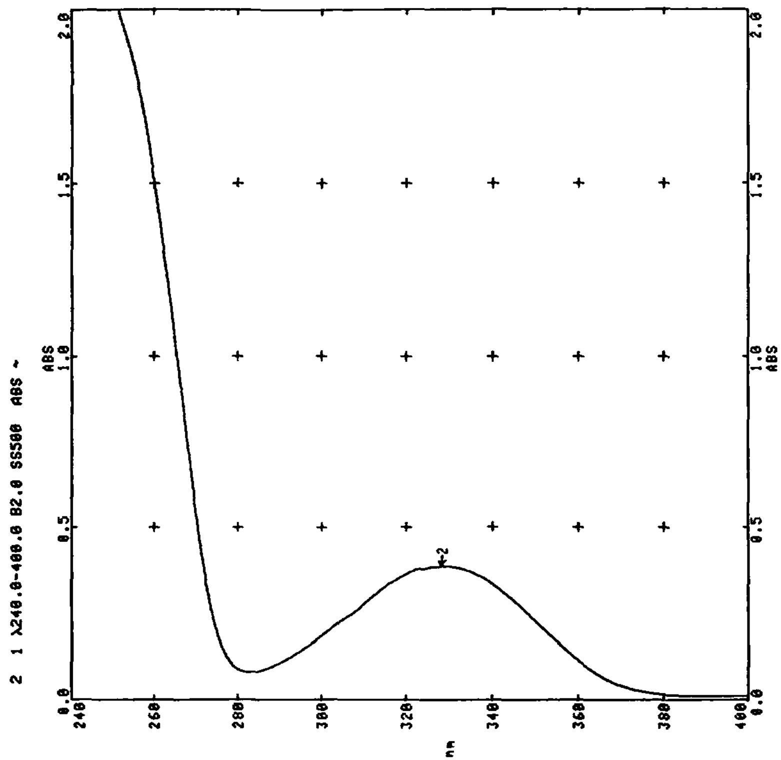


FIGURE 8. Ultraviolet Spectrum of Acebutolol in Chloroform.
Instrument: PU8700 series scanning UV/VIS spectrophotometer.

(37 °C, pH 7.4) (7). Others have reported distribution coefficients of 0.974 and 1.008 after 2 and 6 hours, respectively, in n-octanol/phosphate buffer (pH 7.4, 37 °C) (8).

3.8 Color Tests

The tests and resultant colors have been reported (5) as: methanolic potassium hydroxide, yellow; Nessler's reagent, orange (slow); sulphuric acid, yellow.

3.9 Solubility

Acebutolol has a relatively low lipid solubility (9). Acebutolol hydrochloride has reported solubilities of 200 mg/mL in water and 70 mg/mL in ethanol at room temperature (3). The hydrochloride salt is freely soluble in water.

4. METHODS OF ANALYSIS

4.1 Colorimetric Analysis

Acebutolol has been analyzed utilizing a colorimetric assay (10). Briefly, this method is as follows:

> Acebutolol was extracted from plasma after addition of glycine buffer (pH 10) and ethyl acetate. The organic layer was extracted twice with 3 M sulfuric acid and the two acid extracts were combined and heated at 99 °C for 2 hours. This latter step resulted in the hydrolysis of the butyramide group which, therefore, generated the aromatic amine. Water was added to this solution and the mixture was cooled to 4 °C. After cooling, a 1% (w/v) solution of sodium nitrite was added, followed by the addition of ammonium sulphamate (5% w/v). This solution was shaken at intervals over 15 minutes. A final colorless solution of naphthylethylenediamine dihydrochloride (1% w/v) was added to the mixture and allowed to stand at room temperature for 2 hours. During this time, full color development of the mixture occurred. Absorbance was determined at 565 nm using a scanning spectrophotometer over the range of 400-700 nm.

The reported sensitivity of the assay (0.1 ug/mL) is insufficient for routine clinical monitoring of acebutolol after administration of conventional doses. Furthermore, the assay was non-specific, as both acebutolol and its major metabolite, diacetolol, were detected but not separated from one another.

4.2 Radioimmunoassay

Antisera against acebutolol was produced in rabbits immunized with drug conjugated with bovine serum (11). Radioactive tracer (tritiated acebutolol) was produced by May and Baker (specific activity = 59.6 Ci/mmole). The reported sensitivity was 2.97 x 10^{-9} mol/L of acebutolol.

4.3 Chromatographic Analysis

4.3.1 Thin-Layer

Various systems have been used to detect acebutolol (12,13). The thin-layer chromatographic analyses have been summarized in Table III.

4.3.2 Gas

Gas chromatography has been utilized for the analysis of acebutolol (6,14,15). These methods may lack convenience, as multiple extraction steps contribute to somewhat laborious sample preparation. When applied to analysis of acebutolol in urine, the reported gas chromatography methods are essentially initial screening procedures (qualitative, as opposed to quantitative) based on the following conditions:

1. Hewlett-Packard Series 5790 A gas chromatograph with H-P capillary column (12 m x 0.2 mm I.D., cross-linked methylsilicone), 0.33 um film thickness. The temperature was programmed from 100 to 310 °C at 30 °C/min. The injection port temperature was 270 °C and the carrier gas was helium at a flow-rate of 2 mL/min. Mass spectrometry was used for detection (6).
2. Carlo Erba HRGC 5160 MEGA gas chromatograph with a

TABLE III. Rf Values of Acebutolol under Various Thin-Layer Chromatographic Conditions.

Plate	Solvent System	Rf Value	Ref.
1. Silica Gel 60 F254 (Merck)	Ethyl acetate: methanol: 30% ammonia (85:10:5)	41[a]	12
2. Silica Gel 60 F254 (Merck)	Cyclohexane: toluene: diethylamine (65:25:10)	0[a]	12
3. Silica Gel 60 F254 (Merck)	Ethyl acetate: chloroform (50:50)	0[a]	12
4. Silica Gel 60 F254 (Merck)	Acetone dipped in 0.1 M potassium hydroxide methanolic solution	6[a]	12
5. Silica Gel, 0.22 mm (Polygram Sil N-HR)	Ethyl acetate: methanol (40:5)	0.0	13
6. Silica Gel, 0.22 mm (Polygram Sil N-HR)	Ethyl acetate: methanol: ammonia (sp.gr. 0.88) (40:5:5)	0.73	13

[a]Corrected value (see ref. 12).

J & W Durabond 1 capillary column (30 m x 0.25 mm I.D.), film thickness of 25 um. Temperature was programmed as 1 minute isothermal at 90 °C, and then 40 °C/min to 260 °C. This was followed by a final isothermal period for 15 minutes at 260 °C. Mass spectrometry was used for detection (14).

The analysis of acebutolol in plasma has been reported using a gas chromatographic technique (15). Unlike the methods (above) reported for urine, this method can be used for determination of acebutolol in a quantitative sense. Briefly, the method (which offered sensitivity to approximately 25 ng for acebutolol), was reported using a Hewlett-Packard 5710A series gas chromatograph with a nickel 63 electron capture detector. The column was glass (6 foot, 2 mm I.D.), packed with 3% Dexsil 410 on 100-120 mesh gas-chrom Q. The carrier gas was 5% methane in argon set at a flow of 40 mL/min. The injection port was 250 °C, oven was 275 °C, and the detector was set at 300 °C (15).

4.3.3 High-Performance Liquid

a. Nonstereospecific. There have been numerous reported methods for the analysis of acebutolol using high-performance liquid chromatography (16-22). These methods typically involve reversed-phase chromatography, isocratic flow, and ultraviolet detection. Table IV summarizes the chromatographic conditions used for detection of acebutolol.

b. Stereospecific. The enantiomers of acebutolol have been separated and quantified in biological samples using high-performance liquid chromatography (23-25). The methods are based on pre-column derivatization with a homochiral reagent, and are summarized as follows:

1. Reversed-phase columns (C_{18}) utilizing a mobile phase consisting of methanol:water (62:38) at a flow rate of 1.2 mL/min. The chiral derivatizing reagent was (S)-(-)-1-phenylethyl isocyanate. The diastereoisomers were detected using fluorescence detection at 209 and 320 nm, excitation and emission, respectively. The sensitivity of the assay for acebutolol enantiomers was not reported (23).
2. Reversed-phase columns (ODS2) utilizing a mobile phase consisting of water:methanol:triethylamine

TABLE IV. Chromatographic Conditions of Reported HPLC Assays.

	Column	Mobile Phase	Detector	Ref.
1.	C_2 10-um 250 x 4.6 mm (I.D.)	Methanol: 0.0005 M HCl in 0.05 M NaCl (35:65) (1.2 mL/min)	UV, 254 nm (fixed) UV, 220 nm (variable)	16
2.	Spherisorb ODS 5-um 250 x 3.9 mm (I.D.)	Acetonitrile: 0.1 M phosphate buffer (pH 3.3): water (55:6:39) (1 mL/min)	UV, 240 nm	17
3.	Varian Micro-Pack MCH-10 250 x 2.0 mm (I.D.)	0.01 M dodecyl sodium sulfate in water adjusted to pH 3.5 with glacial acetic acid (Pump A), and same as Pump A, with addition of methanol (50%) (Pump B) (0.67 mL/min)	UV, 240 nm	18
4.	Radial-Pak C_{18} 10-um 100 x 8 mm (I.D.)	Methanol:water (75:25) (1.8 mL/min)	UV, 248 nm	19
5.	LiChrosorb C_{18} 7-um 250 x 4 mm (I.D.)	<u>Phase I</u> Methanol:0.02 M sodium acetate buffer (pH 5.8) (80:20) (1.5 mL/min)	Amperometric, operated at +0.7 V *versus* saturated calomel reference electrode	20

(continued)

TABLE IV. (continued)

	Column	Mobile Phase	Detector	Ref.
		Phase II Acetonitrile: 0.02 M sodium acetate buffer (pH 5.8) (80:20) (1.5 mL/min) Phase III Ethanol:0.02 M sodium acetate buffer (pH 5.8): dichloromethane (90:5:5) (1.0 mL/min)		
6.	LiChrosorb Si 7-um 150 x 4.0 mm (I.D.)	Phosphate buffer (pH 2.2) with increasing organic modifier (1 mL/min)	UV, 270 nm	21
7.	Spherisorb ODS 5-um 250 x 4.6 mm (I.D.)	6% of 0.1 M phosphate buffer (pH 4.0) and 55% aceto-nitrile (1.0 mL/min)	UV, 243 nm	22

(50:50:0.05) at a flow rate of 1.2 mL/min. The chiral derivatizing reagent was (R)-(+)-1-phenylethyl isocyanate. Fluorescence detection was set at 238 and 450 nm for excitation and emission, respectively. The reported sensitivity was approximately 0.05 ug/mL for each enantiomer (24).

3. Normal-phase columns (silica) utilizing a mobile phase consisting of hexane:chloroform:methanol (63:35:2) at a flow rate of 2.0 mL/min. The chiral derivatizing reagent was S-(+)-1-(1-naphthyl)ethyl isocyanate. Fluorescence detection was set at 220 and 389 nm, excitation and emission, respectively. The reported sensitivity was 10 ng/mL for each enantiomer (25).

5. PHARMACOKINETICS

5.1 **Absorption**

Acebutolol is absorbed rapidly, resulting in peak plasma concentrations being achieved within 2-4 hours (26-28). The presence of food or alcohol does not significantly alter the absorption profile of acebutolol (29). Although approximately 90% of an oral dose is absorbed (30), the extent of systemic availability is only between 35% and 45% after administration of drug *via* this route (9). This finding has been explained by the extensive first-pass (hepatic) removal of acebutolol, which contributes to overall drug clearance.

5.2 **Distribution**

Acebutolol is weakly bound to plasma proteins (11-19%) (31). The volume of distribution at steady-state has been reported as between 1.0 to 1.2 L/kg after intravenous dosing (32). After oral dosing, the reported volume of distribution of acebutolol enantiomers was 11.3±3.5 and 9.2±2.6 L/kg for R- and S-acebutolol, respectively (26). Although these two studies reported different volumes of distribution, the differences were likely attributable, in part, to: 1) measures of enantiomer *versus* total (R- plus S-acebutolol) concentrations, and 2) differing methods

of calculating volume terms, where the latter report on acebutolol enantiomers did not factor the amount of drug reaching the systemic circulation after oral dosing.

Following intravenous administration of acebutolol, brain concentrations were similar to atenolol, and less than those reported for propranolol, metoprolol, and oxprenolol (9). These findings were consistent with the drug's physicochemical properties (i.e., low protein binding, low lipophilicity).

5.3 Metabolism

After oral administration of acebutolol to man, the drug is extensively metabolized upon first-pass through the liver. The main metabolite that is formed *via* this first-pass has been identified in man as diacetolol, which has a potency similar to that of acebutolol (27,33-35). Prior to formation of this major metabolite, however, the butyramido group of acebutolol is initially hydrolyzed to form acetolol, a primary amine (36). Subsequently, acetolol undergoes N-acetylation to form the diacetolol metabolite (9). It has been suggested that this first-pass formation of diacetolol is stereoselective for the R-(+)-enantiomer (26). The structures of acebutolol, acetolol and diacetolol have been shown previously (see Figure 1).

The N-acetylation of acetolol has been reported to be independent of acetylator status (34). In elderly subjects, plasma concentrations of acebutolol and diacetolol are significantly higher than in young subjects, which may be a function of either a decreased first-pass metabolism and/or decreased volume of distribution (37).

5.4 Excretion

Acebutolol has a terminal elimination half-life ranging from approximately 3 hours (28) to 8 hours (37,38). Renal excretion of acebutolol and diacetolol together accounted for approximately 25% to 45% of an orally administered dose of acebutolol, whereas after intravenous dosing renal excretion of acebutolol accounted for 40% to 60% of the administered dose (9). In a study of patients with reduced renal function, urinary excretion of both acebutolol and diacetolol was diminished (38). However, the terminal half-life of elimination of acebutolol was unchanged in renal insufficiency. On the contrary, the terminal

half-life of diacetolol was prolonged in patients with reduced renal function (35). Consequently, it was assumed that a non-renal compensatory mechanism of elimination of acebutolol existed. It has further been suggested that non-renal clearance may, in part, consist of biliary and/or intestinal excretion of acebutolol (39,40). More recently, it was determined that the non-renal clearance of acebutolol is stereoselective (26,41).

Acebutolol is excreted into breast milk, as plasma levels of the drug were detected in newborn infants born to hypertensive women treated with acebutolol (42). Acebutolol is excreted into saliva and cerebrospinal fluid, although concentrations of acebutolol in cerebrospinal fluid are much lower than those found in plasma (43).

ACKNOWLEDGEMENTS

The authors are indebted to Drs. F.M. Pasutto, R.T. Coutts, E.E. Knaus and M. Hussain for their contributions to the profile.

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ANALYTICAL PROFILE OF AMOBARBITAL

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

a) 5-Ethyl-5-(3-methylbutyl)-2,4,6-(1H,3H,5H) pyrimidinetrione. (1)
b) 5-Ethyl-5-isopenthylbarbituric acid (1,2,3,4,5).
c) 5-Ethyl-5-isoamylbarbituric acid (1).
d) 2,4,6 (1H, 3H, 5H)-pyrimidinetrione, 5-ethyl-5-(3-methylbutyl). (4)
e) 5-Isoamyl-5-ethylbarbituric acid.
f) Acide isopentyl-5-ethylbarbiturique (6).

1.1.2 Generic Names

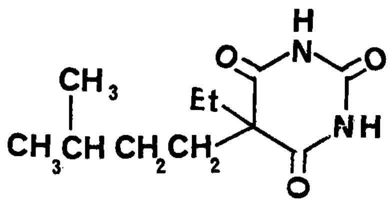
Amylobarbitone, Amal, Amasust, Amystal, Amylobarb, Amytal, Amobarbitalum, Amobarbital, Amobarbitale, Amsal, Amycal, Amydorm, Amytal, Barbamyl, Barbamil, Dormytal, Etamyl, Euntoctal, Isoamitil, Isobec, Isonal, Isomytal, Mylodorm, Neur.Amyl, Placidel, Pentymal, Pentymalum, Sednotic, Stadadorm, Somnal, Sedantite, Sedante, Trasital.

1.2 Formulae

1.2.1 Empirical

$C_{11}H_{18}N_2O_3$

1.2.2 Structural



1.2.3 CAS Registry Number

57-43-2

1.3 Molecular Weight

226.27

1.4 Elemental Composition

C 58.39%, H 8.02%, N 12.38%, O 21.21%.

1.5 Appearance, Color, Odour and Taste

A white odourless crystalline, powder with a slightly bitter taste. (2)

2. Physical Properties

2.1 Melting Range

156-158°. (1)

2.2 Solubility

One gm dissolves in 1300 ml water, in 5 ml alcohol, in 17 ml chloroform, in 6 ml ether. Freely soluble in benzene, soluble in alkaline solutions. Insoluble in petroleum ether, aliphatic hydrocarbons (1). It dissolves in aq. solutions of alkali hydroxides and carbonates. (3)

2.3 pH

A saturated aqueous solution is acidic to litmus paper (1) saturated solution in H_2O has a pH of about 5.6 (2).

2.4 Hygroscopicity

It is hygroscopic (6) it absorbs significant amounts of moisture at 25°, relative humidities upto about 90%. (5)

2.5 Dissociation Constant

The drug has a pKa (Dissociation constant) 8.0 (25°).

2.6 X-Ray Powder Diffraction of Amobarbital Sod.

The X-ray diffraction pattern of amobarbital sod. was determined by a Philips full automated X-ray diffraction spectrogoniometer equipped with PW 1730/10 generator (8). Radiation was provided by a copper target (Cu anode 2000 w, γ= 1.5480 Å) and high intensity x-ray tube operated at 40 KV and 35 MA. The monochromator was a curved single crystal one (PW 1752/00). Divergance slit and the receiving slit were 1 and 0.1° respectively. The scanning speed of the goniometer (PW 1050/81) used was 0.02-2θ per second. The instrument is combined with Philips PM 8210 printing recorder with both analogue recorder and digital printer. The goniometer was aligned using silicon sample before use. The X-ray pattern of amobarbital sod. is presented in Fig. (1). The interplanner distance dÅ and relative intensity I/I_o are shown in Table (1).

2.7 Spectral Properties

2.7.1 Ultraviolet Spectrum (UV)

The UV spectrum of amobarbital in 0.05 M Borax buffer (pH 9.4) and 0.1 M sodium hydroxide (pH 13) (Fig. 2) was scanned from 220 to 340 nm using DMS 90 Varian spectrophotometer (8). It exhibited the following UV data (Table 2).

Table (2). UV characteristics of amobarbital

λ_{max} nm	A(1%, 1 cm)
240	445
252	364

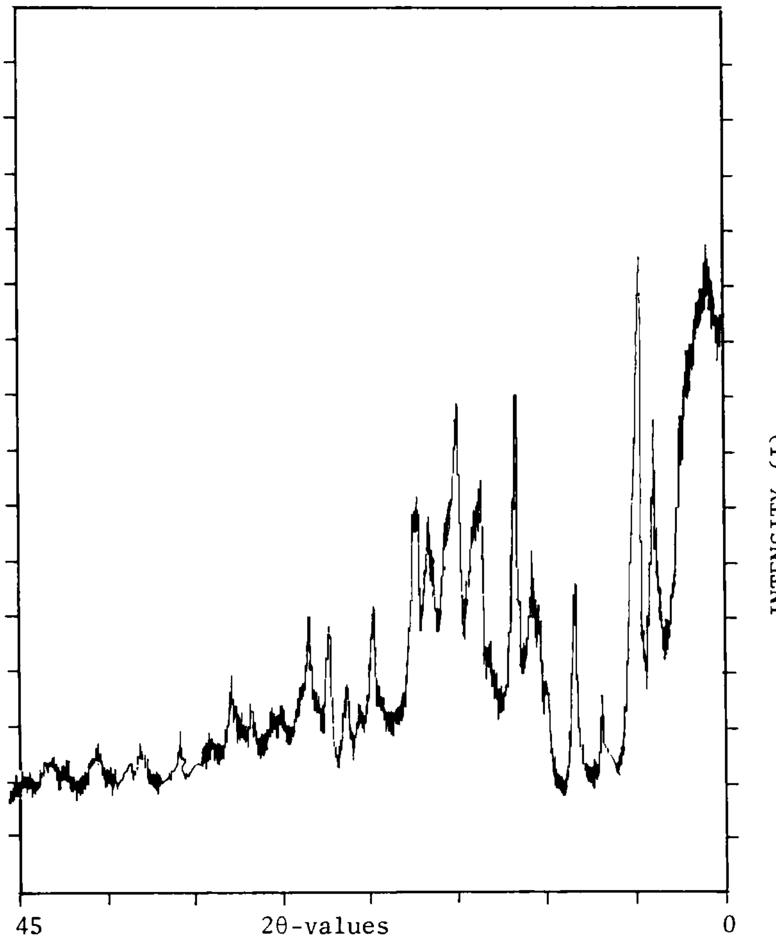


Fig. 1: Characteristic principal lines of the X-ray powder diffraction of amobarbital.

Table 1: X-ray diffraction lines of amobarbital sod.

2θ	dÅ	I/Io %
4.872	18.1367	100
5.927	14.9108	81.631
6.243	14.1581	72.106
6.243	14.1581	72.106
7.185	12.3031	46.369
7.714	11.4598	73.364
8.718	10.1428	97.699
10.379	8.5229	30.481
11.954	7.4032	46.908
13.518	6.5499	31.056
13.518	6.5499	31.056
13.963	6.3425	41.876
14.104	6.1489	49.568
15.494	5.7190	77.318
16.740	5.2958	38.569
17.432	5.0871	63.407
17.768	4.9918	57.872
18.808	4.7179	61.718
19.268	4.6064	57.836
20.186	4.3989	52.552
20.948	4.2406	60.819
23.398	3.8018	43.853
24.091	3.6940	29.439
24.813	3.5882	31.020
25.924	3.4368	40.977
26.661	3.3435	30.301
27.070	3.2939	42.092
28.544	3.1271	27.534
29.016	3.0773	27.749
30.313	2.9485	28.540
31.417	2.8473	32.674
35.022	2.5621	22.933
36.570	2.4571	22.789

I/I_o = relative intensity (based on highest intensity as 100).
dÅ = interplanner distance.

UV scan of amobarbital sodium in H_2O is also presented in Fig. (2A). It exhibited λ_{max} at 207 nm and 240 nm. The scan was obtained on LKB 4054 UV/Vis spectrophotometer (8).

2.7.2 Infrared Spectrum (IR)

The IR spectrum of amobarbital as KBr disc was recorded on a Perkin Elmer 580 B infrared spectrometer to which an infrared data station is attached (Fig. 3). The structural assignments have been correlated with various frequencies (Table 3). (8)

Table (3). IR characteristics of amobarbital

Frequency cm^{-1}	Assignment
2800-2950	Aliphatic C-H
3000-3200	C-NH
1696	NH\C = O
1428	NH band
842	C-C strech
400-600	- Alkyl

2.7.3 Nuclear Magnetic Resonance Spectra

2.7.3.1 Proton Spectrum

The PMR spectrum of amobarbital in DMSO-d_6 (Fig. 4) was recorded on a Varian (FT80 A) NMR spectrophotometer using TMS as internal standard (8). Chemical shifts are shown in Table 4.

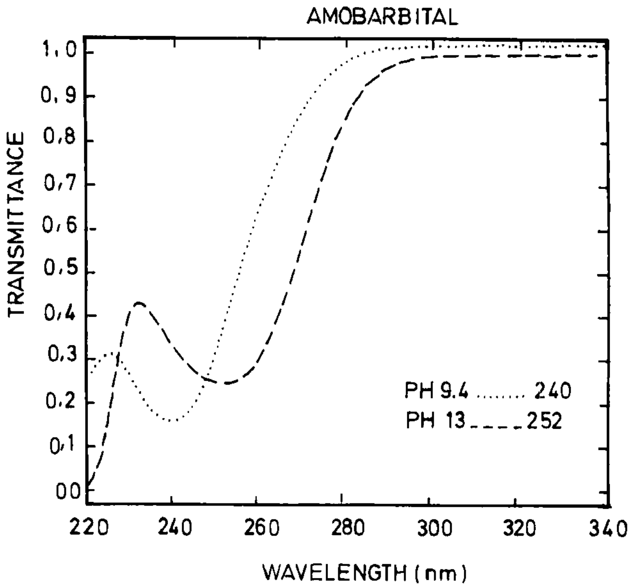


Fig. 2: UV spectrum of amobarbital.

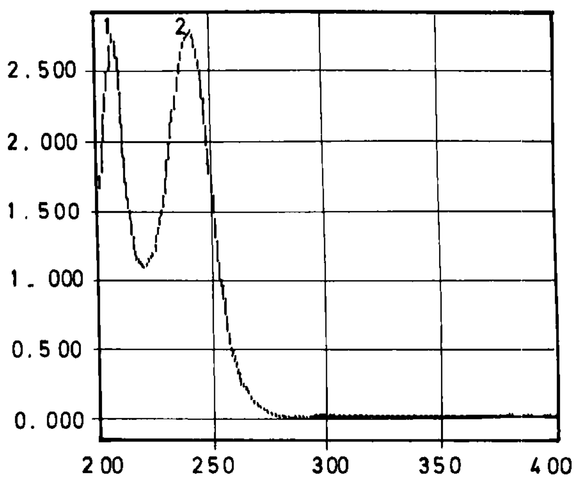


Fig. 2A: UV spectrum of amobarbital sod. in H_2O.

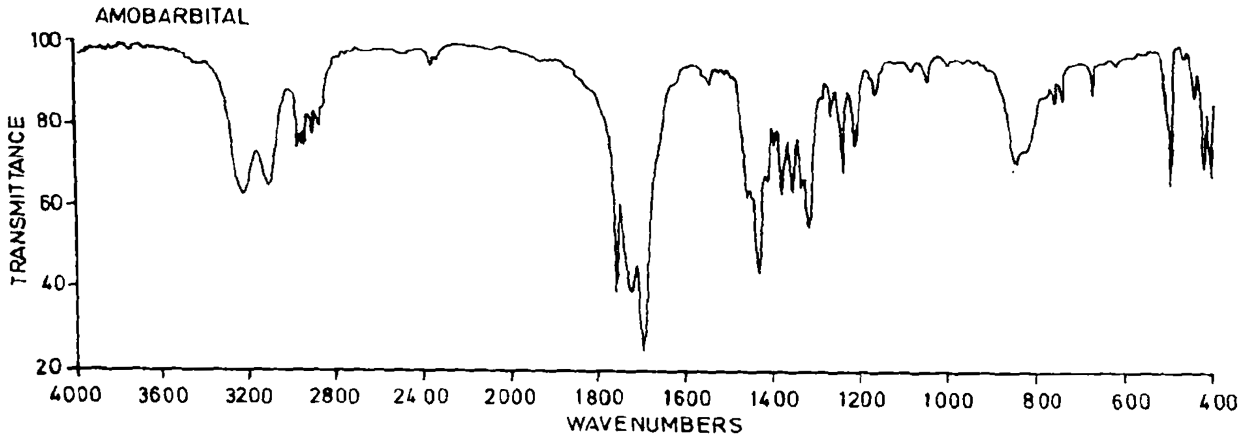


Fig. 3: I.R. spectrum of amobarbital.

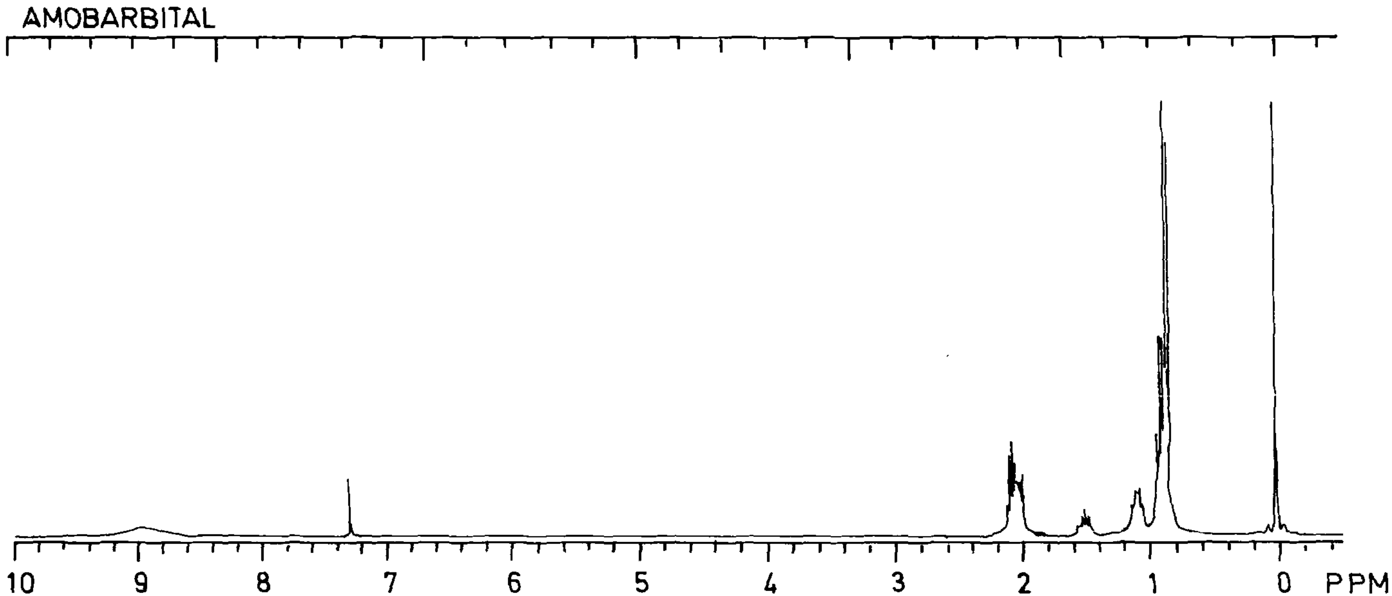


Fig. 4: PMR spectrum of amobarbital.

Table (4). PMR characteristics of amobarbital

Group	Chemical shift (δ) ppm
CH_3-C	0.9 m
C-CH_2-C-	1.1
C-CH-C C	1.5
- NH	2.1

2.7.3.2 ^{13}C NMR Spectra

The ^{13}C-NMR complete decoupled and off-resonance spectra of amobarbital in DMSO-d_6, were recorded on Joel FX-100 NMR spectrometer (8) using TMS as internal reference. Various carbon chemical shifts are shown in the Figs. 5 and 6.

2.7.4 Mass Spectrum

The mass spectrum of amobarbital (8) was obtained ( ) by direct injection of the sample into a Finigan-Mat 1020 GC/Mass spectrometer. The ionization beam energy was 70 eV. Figure (7) is a bar graph of the mass spectrum. Identification of the prominent masses is presented in (Table 5).

3. Synthesis

A general procedure (9) for the synthesis, of amobarbital is presented. Monochloroacetic acid is treated with sodium cyanide; the resulting cyanoacetic acid is treated with hydrochloric acid in the presence of ethanol, yielding the diethyl ester of malonic acid. The ester, in absolute alcohol, solution, is treated with the theoretical quantity of metallic sodium to replace one hydrogen of the CH_2 group and thereupon a slight excess of the theoretical amount of ethyl bromide, is added. The second hydrogen is similarly replaced by using iso-amyl bromide. The diethyl ester of ethyl, isopentyl malonic acid thus obtained is heated in an alcoholic solution, in the presence of sodium and urea to form amobarbital.

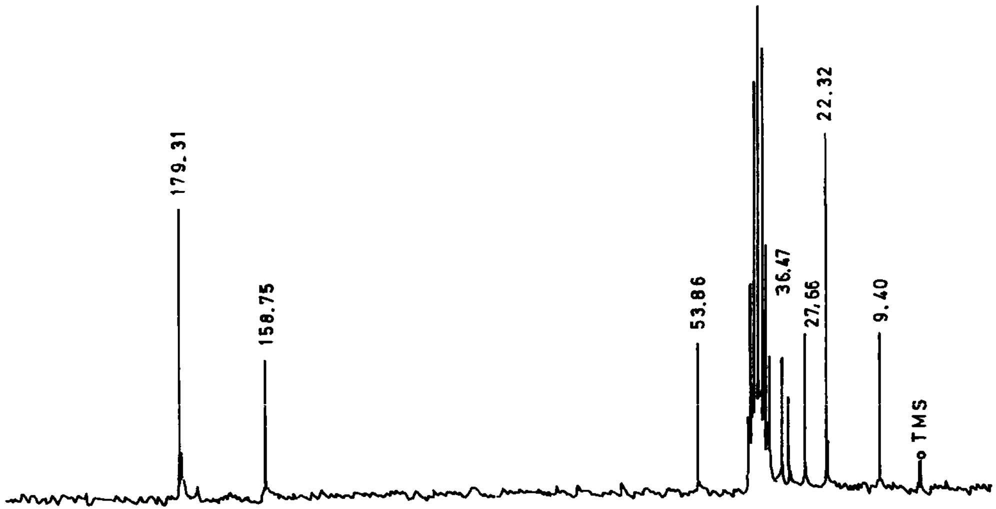


Fig. 5; ^{13}C - NMR NOISE - DECOUPLED SPECTRUM OF AMOBARBITAL

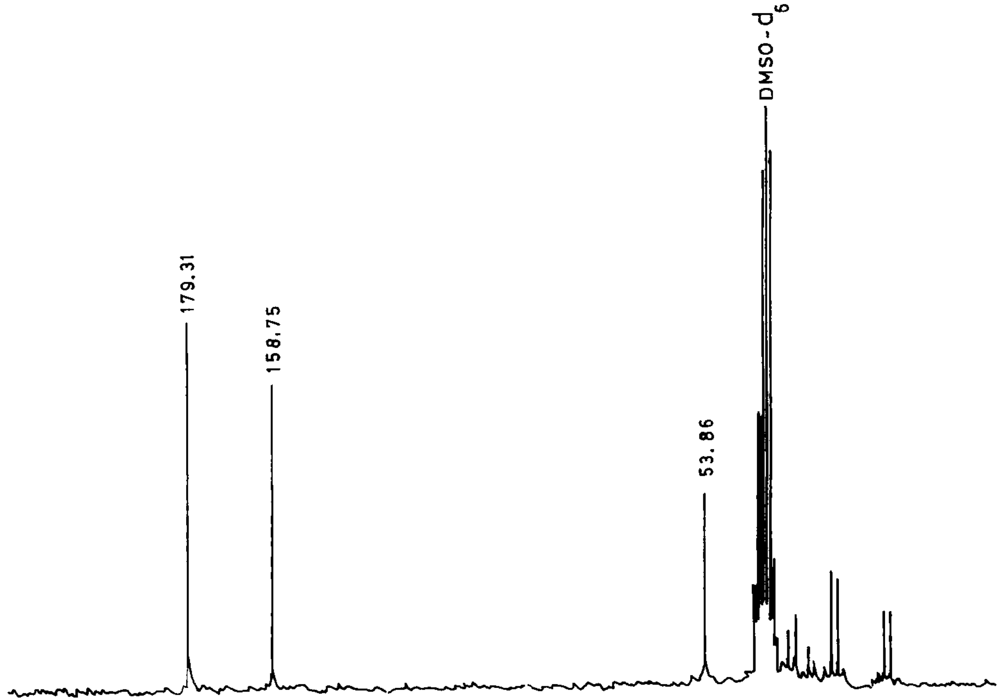


Fig. 6: ^{13}CNMR OFF-RESONANCE SPECTRUM OF AMOBARBITAL

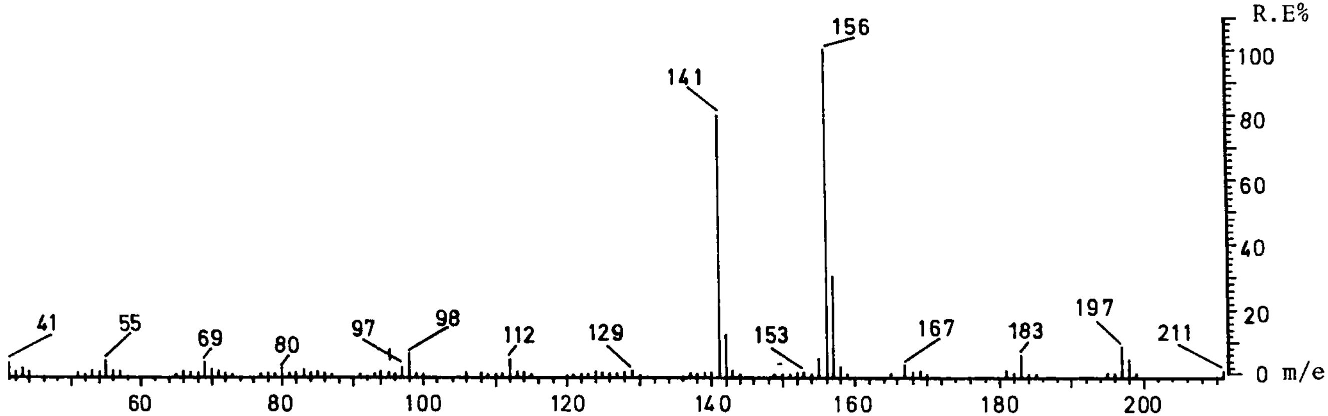


Fig. 7: Mass spectrum of amobarbital.

Scheme:

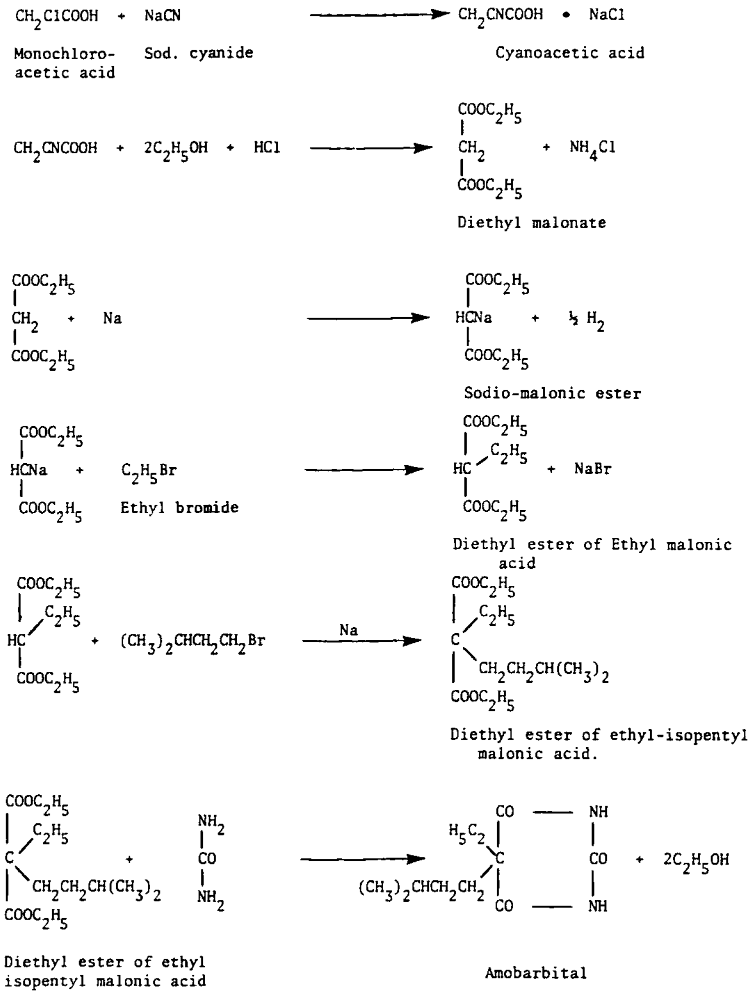
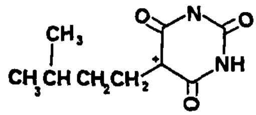
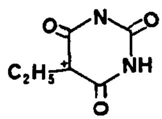
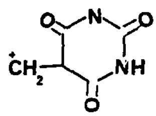

CH2ClCOOH + NaCN → CH2CNCOOH • NaCl
Monochloro-acetic acid
Sod. cyanide
Cyanoacetic acid
CH2CNCOOH + 2C2H5OH + HCl
+ NH4Cl
Diethyl malonate
Na
HCNa + ½ H2
Sodio-malonic ester
C2H5Br
Ethyl bromide
+ NaBr
Diethyl ester of Ethyl malonic acid
(CH3)2CHCH2CH2Br
Na
Diethyl ester of ethyl-isopentyl malonic acid.
Diethyl ester of ethyl isopentyl malonic acid
+ 2C2H5OH
Amobarbital

Table (5). The most prominent fragments of amobarbital

m/e	Relative intensity %	Fragment
197	8	
156	100	
141	78	

4. Metabolism

Amobarbital is readily absorbed after oral administration (5). After an oral dose of 20 mg of the sod.-salt, a plasma concentration of 2 µg/ml is attained after 4 hours and this drops to about 1 µg/ml after 24 hours.

Plasma amobarbital concentrations of greater than 50 µg/ml usually produce deep coma and are potentially lethal (7). The estimated minimum lethal dose is 1.5 g (10).

Amylobarbitone crosses the placenta, is secreted in the milk, and is secreted in the saliva in concentrations with parallel those in the serum. The 3-hydroxy metabolite accumulates in renal function impairment; volume of distribution about 70 litres (5).

Amylobarbital is metabolized by the liver *via* penultimate oxidation of the 3′-methylbutyl

substituent to form a tertiary alcohol, hydroxyamobarbital, which is an inactive metabolite. About 40-50% of an oral hypnotic dose of amobarbital is excreted in urine as hydroxyamobarbital and its glucuronide conjugates. Less than 1% of an oral hyponotic dose of the drug is excreted in urine unchanged. Conjugates of hydroxyamobarbital excreted in feces or urine and or further oxidation products not yield identified, may account for the remainder of the dose (7).

In 6 days, 80-90% of a dose is excreted in the urine and 4-5% is excreted in the faeces; of the excreted material, 30-50% is 3′-hydroxyamylobarbitone and 10-30% appears to be N-hydroxyamylobarbitone; less than 1% is excreted as unchanged drug in the urine (5).

5. Toxicity

LD_{50} (lethal dose) of amobarbital (oral) (10) in rabbits is 575 mg/kg and in rats 115 mg/kg. The estimated minimum lethal dose in man is 1.5 g; the blood-level associated with severe poisoning is 2 to 4 mg%. When the blood level is in the region of 1.5 to 2 mg% recovery is possible.

<u>Case example</u>: A 51-year old man swallowed 20 g of amylobarbitone. Twelve hours after ingestion the blood level was 16 mg%. He recovered after dialysis (11).

A 77 year old woman was found dead after ingesting about fifty-five (200 mg) amylobarbitone capsules in 4 days. Post-mortem levels were: blood, 16.3 mg%; brain, 11.9 mg%; liver, 36.2 mg%; stomach contents, 3030 mg; urine, 0.7 mg%. The blood level is stated to be one of the highest ever recorded (10).

6. Uses

Amobarbital and amobarbital sodium are used principally as hypnotics in the short-term treatment of isomnia for periods up to 2 weeks duration. Barbiturates appear to loose their efficiency for sleep induction and maintenance after this period of

time. The drugs are also used for routine sedation and to relieve anxiety and provide sedation pre operatively (7).

The drug may be used IV or IM to control status epilepticus or acute seizure episodes resulting from meningitis, poisons, eclampsia, tetanus, or chorea. The drug has also been used parenterally to control acute episodes of agitated behaviour in psychoses such as catotonic, negetivistic, or manic reactions, but has little value in long-term management of psychoses. Parental amobarbital sodium may also be useful in narcoanalysis, narcotherapy and as a diagnostic aid in schizophrenia (7).

Drug is given by mouth in a single dose of 100-200 mg as a hypnotic. Upto 400 mg may be given daily in divided doses as a sedative, the usual dose, however, being 30-60 mg daily (5).

7. Cautions

IV administered drug may cause respiratory depression, apnea, or hypotension, particularly if the drug is administered too rapidly. The drug must be administered slowly at a rate not greater than 100 mg/minute and personnel and equipment should be readily available for administration of artificial respiration.

Hypnotic doses should be taken half to one hour before bedtime. The tablets may cause drowsiness on the following day. Persons effected should not drive or operate machinery. Alcohol should be avoided (5).

8. Methods of Analysis

8.1 Elemental Analysis

C 58.39%, H 8.02%, N 12.38%, O 21.21%.

8.2 Identification

(a) A saturated solution is acidic to litmus paper (3).

(b) Boil 0.2 g with 10 ml of 1N sodium hydroxide ammonia is evolved (4).

(c) Incinerate about 0.1 g: the residue, when moistened with hydrochloric acid and introduced on a platinum wire into the flame of a Bunsun burner gives a yellow color to the flame (12).

(d) A solution of 0.25 g in 5 ml of H_2O is alkaline to litmus solution, on acidification with 2 M hydrochloric acid it yields a white ppt. (12)

(e) Dissolve 50 mg in 2 ml of a 0.2 percent w/v solution of cobalt^{+2} acetate in methanol, warm, add 50 mg of powdered sodium teteraborate, and heat to boiling, a bluish violet color is produced (12).

(f) Dissolve 0.2 g in 5 ml of absolute ethanol, add 10 ml of silver nitrate-pyridine reagent and titrate with 0.1 M ethanol sodium hydroxide vs using 0.25 ml of thymolphthalein solution as indicator, until a full blue color is obtained. Each ml of 0.1 M ethanolsodium hydroxide vs is equivalent to 0.02483 g of $C_{11}H_{17}N_2NaO_3$ (12).

(g) Accurately weighed 450 mg of amobarbital was dissolved in 60 ml of dimethylformamide in a 125-ml conical flask. Add 4 drops of thymol blue TS, and titrate with 0.1 N sodium methoxide vs, using a magnetic stirrer and the precaution is taken against the absorption of atmospheric CO_2. One blank determination is performed. Each ml of 0.1 N sodium methoxide is equivalent to 22.63 mg of $C_{11}H_{18}N_2O_3$ (4).

(h) Triturate 0.6 g with 0.15 g of anhydrous sodium carbonate and 5 ml of water, add a solution of 0.45 g of 4-nitrobenzyl chloride in 10 ml of ethanol (96%) and warm on a water bath for 30 minutes cool, allow to stand for one hour, filter and wash the residue with 10 ml of 1M sodium hydroxide and then with water. The melting point of the residue, after recrystallisation from ethanol (96=%), is about 150° or about 168° (3).

(i) The UV absorption of amytobarbitone in 0.1 N NaOH, is maxima at 220 nm (E1%, 1 cm = 954) and 244 nm (E1%, 1 cm = 365) (5).

(j) Dry it at 105° for four hours it looses not more than 1.0% of its weight (4).

8.3 Titrimetric Method

Determination of amylobarbitone has been carried out by titration in dimethylformamide and dimethylsulphoxide with 0.1 M pot. t.butoxide, pot. isopentoxide or pot. isopropoxide in toluene. The end point was detected potentiometrically (glass electrode vs calomal electrode) or visually with use of different indicators (13).

8.4 Spectrophotometric Methods

8.4.1 UV Absorption Spectroscopy

De Fabrizio made the suspension containing amylobarbitone in 50 ml of ethanol-acetic acid (1:1) centrifuge and apply 10 ml of the supernatent liquid to a column (50 cm X 2 cm) packed with alginic acid (4 g) that has been pre-washed with 2N-HCl and ethanol-acetic acid (1:1) wash the column with ethanol-acetic acid (1:1) at 1 ml/min and collect 100 ml of eluate adjust the pH of 2 ml of solution to 4 and that of second 2 ml portion to 10 with 0.02 N-NaOH, dilute each solution to 50 ml with H_2O and measure the absorbance of the solution at pH 10 and (that of pH 4 in the reference cell) at 285.5 nm (14).

8.4.2 Infrared Spectroscopy

Amylobarbitone can be identified (15) by stirring 100 g of e.g. liver or kidney with H_2SO_4 at pH 2 to 3 for about two hours. Repeat the extraction twice (one hour each time) with 0.01 M - H_2SO_4 at pH 2-3, dilute the combined extracts to known volume with 0.01 M - H_2SO_4, and pass 50 ml of the solution through the column (40 cm X 2.5 cm) of sephadex G 25 (particle size 100-300 µm) and elute with 0.01 M - H_2SO_4. Discard the first 150 ml of eluate. Collect the next 200 ml, and extract the barbiturates therefrom into $CHCl_3$ (3 X 50 ml). Evaporate the combined $CHCl_3$ extracts, dissolve the residue in $CHCl_3$, press 0.5 ml of the solution into a tablet with KBr ($\simeq$ 230 mg), and record the i.r. spectrum from 4000 to 700 cm^{-1}.

Amylobarbitone can be identified from absorption bands in the regions 3200 to 3000 cm^{-1}.

Moss et al. (16) also analysed the drug using infrared spectroscopy.

8.5 Chromatographic Methods

8.5.1 Paper Chromatography

Some paper chromatographic systems used for the determination of amobarbital have been summarised in Table (6).

8.5.2 Column Chromatography

Amylobarbitone can be separated by column chromatography using gel. The fractions eluted from a Sephadex G-25 column by 0.02 N - H_2SO_4 (pH 2.0), universal buffer solution (pH 2.0) phosphate buffer solution (pH 4.0) or saturated aqueous Na_2-B_4O_7, 0.3 N NaOH (3:1) (pH about 10.0) is shown to depend on the pH of the eluent and not on its ionic composition (18).

8.5.3 Gas Chromatography

Different gas chromatographic methods used for the assay of amobarbital are summarised in the Table (7).

8.5.4 Thin-Layer Chromatography

A summary of different TLC methods used for the determination of amobarbital are given in the Table (8).

8.5.5 High Performance Liquid Chromatography

Several methods have been developed for the estimation of amobarbital by HPLC are given in the Table (9).

9. Acknowledgements

The authors wish to thank Mr. Tanvir A. Butt, Pharmaceutical Chemistry Department, College of Pharmacy, King Saud University and Mr. Babikir Awad

Mustafa, College of Applied Medical Sciences, King Saud University, for their secretarial and technical assistance respectively in preparing the manuscript.

Table 6: Parameters used for paper chromatography of amobarbital

No.	Support	Developing solvent	Detection	Reference
1.	Paper Whatman No. 1 impregnated with 10% solution of ($Na_3PO_4.12H_2O$) and dried.	Ethylene dichloride	UV 254 nm	10
2.	Paper Whatman No. 1 impregnated by dipping in 20% solution of formamide in acetone and dried.	5N.NH_4OH: benzene: $CHCl_3$ (6:3:13).	$AgNO_3$ spray	17
3.	Whatman No. 4	Light petroleum	Paper exposed to ammonia vapour and then UV 254 nm.	17

Table 7: Summary of conditions used for GC of amylobarbitone

Column support	Mesh	Temperature	Flow rate	Sample	Reference
Glass column (3 ft X 2 mm) of 10% of UCW-982.	80-100	170°	--	Blood	19
Column packed with OV-17 - OV-101 2:3	--	150-300°	N_2 carrier gas 40 ml/min.	Plasma	20
Glass column (1.8 m X 4 mm) packed with 3% of SP-2250 DA	100-120	210-240°	--		21
Borosilicate-glass column packed with 10% of SE-30 on Chromosorb W AW-DMCS.	--	180°	--	Liver tissues	22

Continued /...

Continued (Table 7...)

Column containing 0.2% of WG-H on Chromosorb W (HP).	--	225°	--	Urine	23
(2.5 m X 0.3 cm) 3% of SE-30 Chromaton N-AW HMDS (0.25 to 0.31 mm).	--	190°	Carrier gas N_2.	Biological Fluid	24
Coiled glass column (6 ft X 2 mm) packed with 3% of SE-30 OV-17) supported on chromosorb W.	--	200° or 230°	--	--	25
3% of SE-30 on Chromosorb 750	--	--	--	Sliva	26
6 Ft column packed with 3% of OV-101 on Chromosorb WHP.	100-120	180°	He as carrier gas	Tissues	27
(25 m X 0.31 mm) of [illegible]	--	190°	N_2 carrier gas	Plasma	28

Table 8: Summary of conditions used for the TLC of amylobarbitone

Plate	Developing solvent	Detection	R_f	Reference
Silica gel	H_2O-methanol-aq. NH_3 (40:10:1).	-	--	29
Silufol sheets	$CHCl_3$-acetone-aq. 22 to 24% NH_3 (25:25:1).	254 UV	6.52	30
Silica gel GF	Ethylacetate-methanol-aq. NH_3 (17:2:1).	UV	--	31

Table 9: Summary of HPLC conditions for the determination of amylobarbitone

Column	Mobile phase	Flow rate	Retention time	Sample	Detection	Reference
(30 cm X 4 mm) μ Bondapak C18.	Methanol:H2O (4:1)	2 ml/min	10 min.	--	249 nm	32
Stainless steel (10 cm X 5 mm) packed with Hypersil ODS (5 μm).	0.1 M-NaH2PO4: methanol (3:2; pH 8.5).	2 ml/min	--	Blood	240 nm	33
Reversed phase (125 cm X 4.5 mm) SAS Hypersil. A guard column (5 cmX 2.1) nm of Co:Pel ODS.	Acetonitrile-5 mM-tetrabutylammonium phosphate solution (1:4).	1.16 ml/min	13.6 min	Plasma	200 nm	34
(30 cm X 4 mm) of μ Bondapak C18.	Acetonitrile-phosphate buffer solution pH 4.4 (43:157).	3 ml/min	--	Serum	195 nm	35

Continued /...

Continued (Table 9...)

Reversed phase column (C18).	Methanol-0.1M-phosphate buffer solution pH (4.o)	--	--	Tissues	210 nm	27
(25 cm X 4 mm) of Nucleosil 7 C18 (7 µm)	0.01M acetate buffer (pH 4.0) acetonitrile (7:3)	--	--	Serum	210 nm	36

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Bupivacaine

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1. Foreword

Bupivacaine is a potent long acting local anesthetic of the amide class which is available with or without epinephrine (as the bitartrate, 1:200,000). It is administered by local infiltration and for peripheral nerve block and caudal and lumbar epidural block. Bupivacaine is also available as a sterile hyperbaric solution containing dextrose for subarachnoid injection (spinal block) (1,2).

2. Description

2.1 Nomenclature

Bupivacaine HCl
Carbostesin
2-Piperidinecarboxamide,1-butyl-N-(2,6-dimethylphenyl)-,monohydrochloride, monohydrate
d,1-1-Butyl-2',6'-pipecoloxylidide monohydrochloride monohydrate

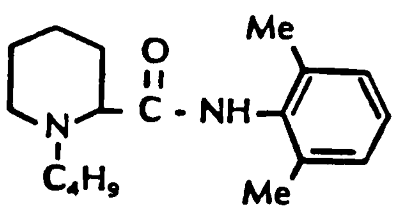
2.2 Formula

$C_{18}H_{28}N_2O \cdot HCl \cdot H_2O$

2.3 Molecular Weight

monohydrochloride monohydrate	342.9
base	288.4

2.4 Structure



2.5 CA Registry Numbers

base	[2180-92-9]
hydrochloride, anhydrous	[18010-40-7]
hydrochloride, monohydrate	[14252-80-3]

2.6 Appearance, Color, Odor

Bupivacaine hydrochloride is a white, odorless crystalline powder.

2.7 Recognized Dosage Forms

The forms of bupivacaine hydrochloride recognized by the U.S.P. include the bulk drug and the following formulations (1,3).

Bupivacaine in Dextrose Injection, 7.5 mg hydrochloride per mL
Bupivacaine and Epinephrine Injection, 2.5 and 5.0 mg hydrochloride per mL
Bupivacaine Hydrochloride Injection, 2.5, 5.0 and 7.5 mg hydrochloride per mL

Synthesis and Resolution

3.1 Synthesis

The synthesis of bupivacaine originally was accomplished in Sweden by Ekenstam and was reported in the literature and in a U.S. patent (4,5). This method is illustrated in Figure 1. Briefly, malonic acid ethyl ester 2,6-xylidide resulted from heating 2,6-xylidine with diethylmalonate. The recrystallized monoxylidide was converted to the isonitrosomalonic ester xylidide which was reduced to the amine. Delta bromobutylaminomalonic ester xylidide was formed following the substitution reaction on its precursor. Finally, the cyclization reaction to bupivacaine base was completed under acidic conditions.

3.2 Resolution

The optical isomers of (+/-) bupivacaine were resolved using (+) tartaric acid in boiling 2-propanol. The (+) isomer precipitated first as the (+) tartrate while the (-) bupivacaine isomer salt of (+) tartaric acid was recovered from the resolution liquor (6).

SYNTHESIS

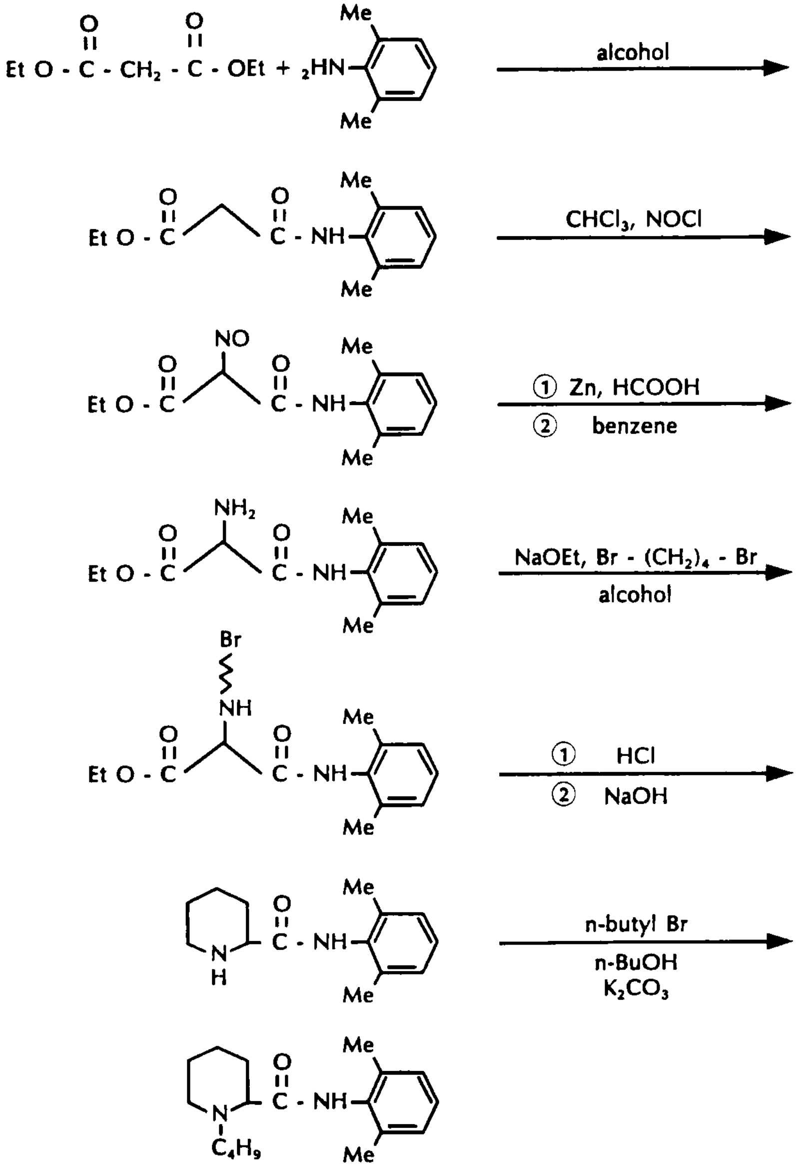


Figure 1. Synthesis of bupivacaine

4. Physical Properties

4.1 Melting Point

The reported melting points of bupivacaine base and salts as well as the resolved isomers are given in Table 1.

4.2 Optical Rotation

Optical rotation results found for resolved optical isomers are shown in Table 2.

4.3 Elemental Analysis

Results for elemental analysis of bupivacaine are shown in Table 3.

4.4 Ionization Constant

The pKa values of bupivacaine and its resolved isomers were determined early by Friberger and Aberg and are listed in Table 4 (10). While these values have been often quoted in the literature, the method used in their determination was not described, although it could have been the solubility method. A more recent potentiometric study revealed similar results along with an enthalpy of ionization of 8.19 kcal/mol for bupivacaine (11).

4.5 Partition Coefficient

The partition coefficients of bupivacaine base isomers have been measured in various systems. Widely divergent results of these studies dependent on the solvents in use are listed in Table 5.

4.6 Solubility

The solubility of bupivacaine hydrochloride has been described as 1 in 25 of water (40 mg/mL), 1 in 8 of alcohol (125 mg/mL) and slightly soluble in acetone, chloroform and ether (2,13). In addition data shown in Table 6 for bupivacaine hydrochloride solubility has been obtained showing the decreased solubility of the racemate compared to the resolved isomers.

Figure 2 shows a pH/solubility profile obtained for (+/-) bupivacaine hydrochloride with the characteristic break in the curve at pH 6, below which further increases in hydronium concentration fail to increase bupivacaine solubility (14).

Table 1
Melting Points of Bupivacaine

Form	Isomer	Melting Point °C	Reference
base	(+/-)	107.5-108.0	4
base	(+/-)	107-108	8
base	(+)	135-137	6
base	(-)	135-137	6
HCl	(+/-)	258.5	4
HCl	(+/-)	258-259	7
HCl	(+/-)	255-256	8
HCl	(+/-)	250-255	9
HCl	S(-)	258	7
HCl	R(+)	260	7
HCl	(+)	258	6
HCl	(-)	255-257	6

Table 2
Optical Rotation of Bupivacaine Isomers

Form	Isomer	α_D^{25}	Preparation	Reference
base	(+)	+ 81	5% in methanol	6
base	(-)	- 80.9	5% in methanol	6
HCl	(+)	+ 12.7	2% in water	6
HCl	(-)	- 12.3	2% in water	6
HCl	S(-)	- 12.0	2% in water	7
HCl	R(+)	- 12.7	2% in water	7

Table 3
Bupivacaine Elemental Analysis

Form	Isomer	% C	% H	% N	% O	Reference
base	(+/-)	74.96	9.78	9.71	5.54	8

Table 4
Bupivacaine Ionization Constants

Isomer	pKa	Reference
(+/-)	8.09	10
(+)	8.09	10
(-)	8.09	10
(+/-)	8.17	11

Table 5
Bupivacaine Base Partition Coefficients

Isomer	Organic / Aqueous	Partition Coefficient	Reference
(+/-)	oleyl alcohol/ water	1565	10
(+/-)	n-heptane/ pH 7.4 buffer	27.5	12
(-)	oleyl alcohol/ water	1624	10
(+)	oleyl alcohol/ water	1603	10

Table 6
Aqueous Solubility of Bupivacaine Hydrochloride

Isomer	Solubility (mg/mL)	pH	Reference
(+/-)	0.83	7.4	12
(+/-)	23*	3	10
(+)	64*	3	10
(-)	64*	3	10

*extrapolated

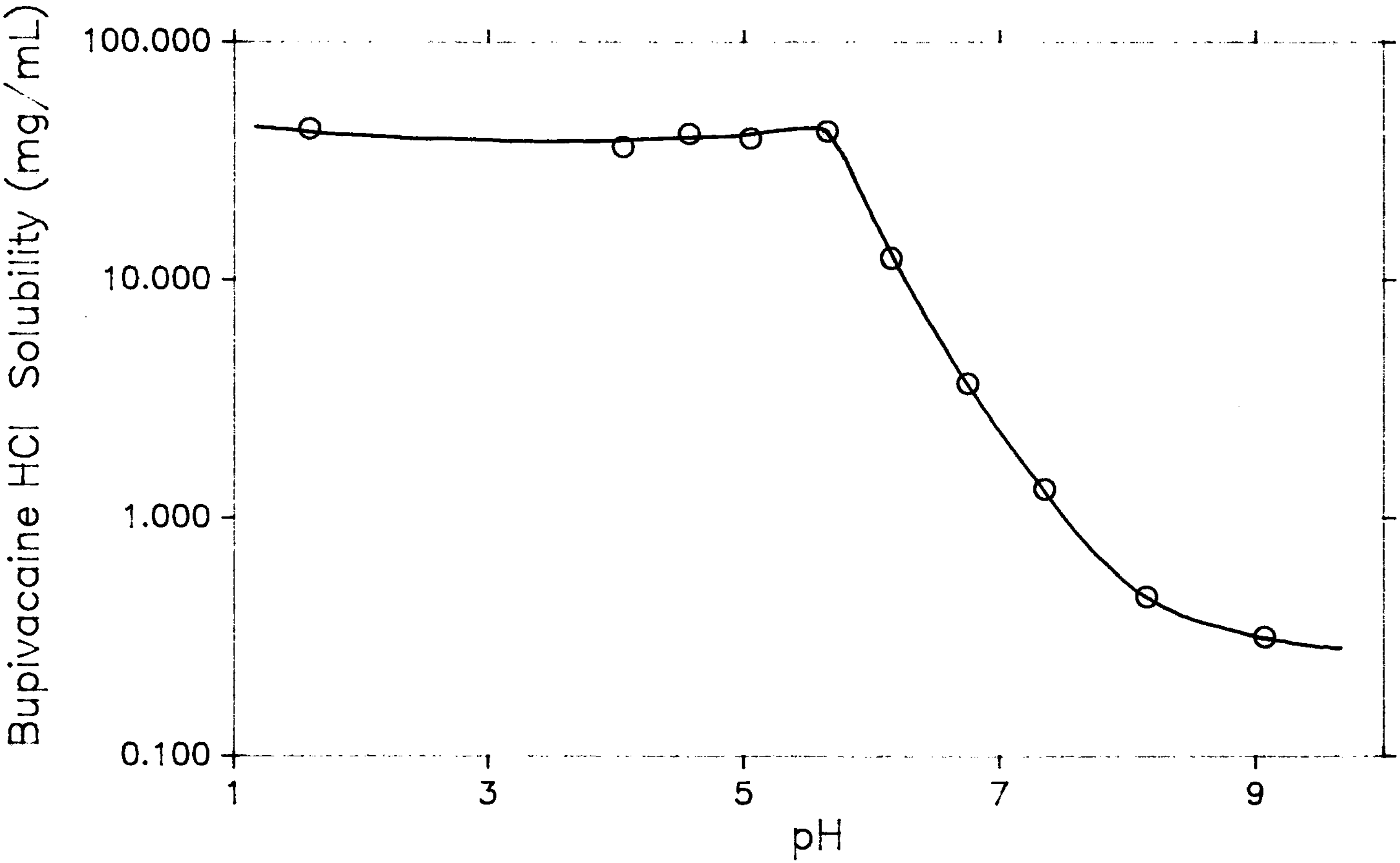


Figure 2. pH/solubility relationship for bupivacaine.

Investigations on the solubility of bupivacaine base have shown an inverse temperature dependence. Results found for solutions in 0.5 M and 0.7 M phosphate buffers (pH 7.4) and in 1-4 mM NaOH (pH 10.43-11.86) are shown in Table 7 where a 1 mM solution is equivalent to 0.288 mg/mL (15, 16).

Little ionic strength effect from 0.1 to 1.5 M was observed on the solubility of (+/-) bupivacaine HCl or the resolved isomers at pH 7.4 and 23°C in another study (10).

4.7 Spectral

4.71 Mass Spectrum

The mass spectrum of bupivacaine is shown in Figure 3 resulting from a methane chemical ionization as obtained on a Nermag R-10-10C mass spectrometer with a direct insertion probe, a 160°C source temperature and an electron energy of 94.4 volts. The molecular ion is seen at m/z 289 (MH^+) while the peaks at m/z 317 and 329 correspond to $MH^+ + C_2H_5$ and $MH^+ + C_3H_4$. The peak at m/z 140 corresponds to the 1-butylpiperidine fragment (17).

4.72 Nuclear Magnetic Resonance Spectrum

The proton magnetic resonance spectrum of bupivacaine is shown in Figure 4. It was obtained on a Jeol GSX 270 for a solution in D_2O. The spectral interpretation is listed in Table 8 which was made possible using chemical shifts, intensities, literature values and 2-dimensional COSY results which are shown in Figure 5 for bupivacaine. Singlets are seen for the two methyl groups on the phenyl ring and the exchangeable protons whereas the aliphatic chain methyl exhibits a triplet. The piperidine methine is a doublet while the nonequivalent methylenes of the piperidine ring appear as a doublet of doublets. Integration of the four exchangeable protons is slightly low as a result of the fast pulse delay (3.0 sec) used in accumulation of the spectrum (17).

The ^{13}C-nmr decoupled spectrum of bupivacaine is shown in Figure 6 as obtained on the same

Table 7
Solubility of Bupivacaine Base

Temp (°C)	Concentration Found (mM) 0.5 M phosphate buffer	0.7 M phosphate buffer	1-4 mM NaOH
14.5	1.35	-	-
14.9	-	-	0.375
25	0.85	0.481	0.318
34.5	-	-	0.313
37	0.57	0.363	-
48	0.465	-	-
54.8	0.374	-	-

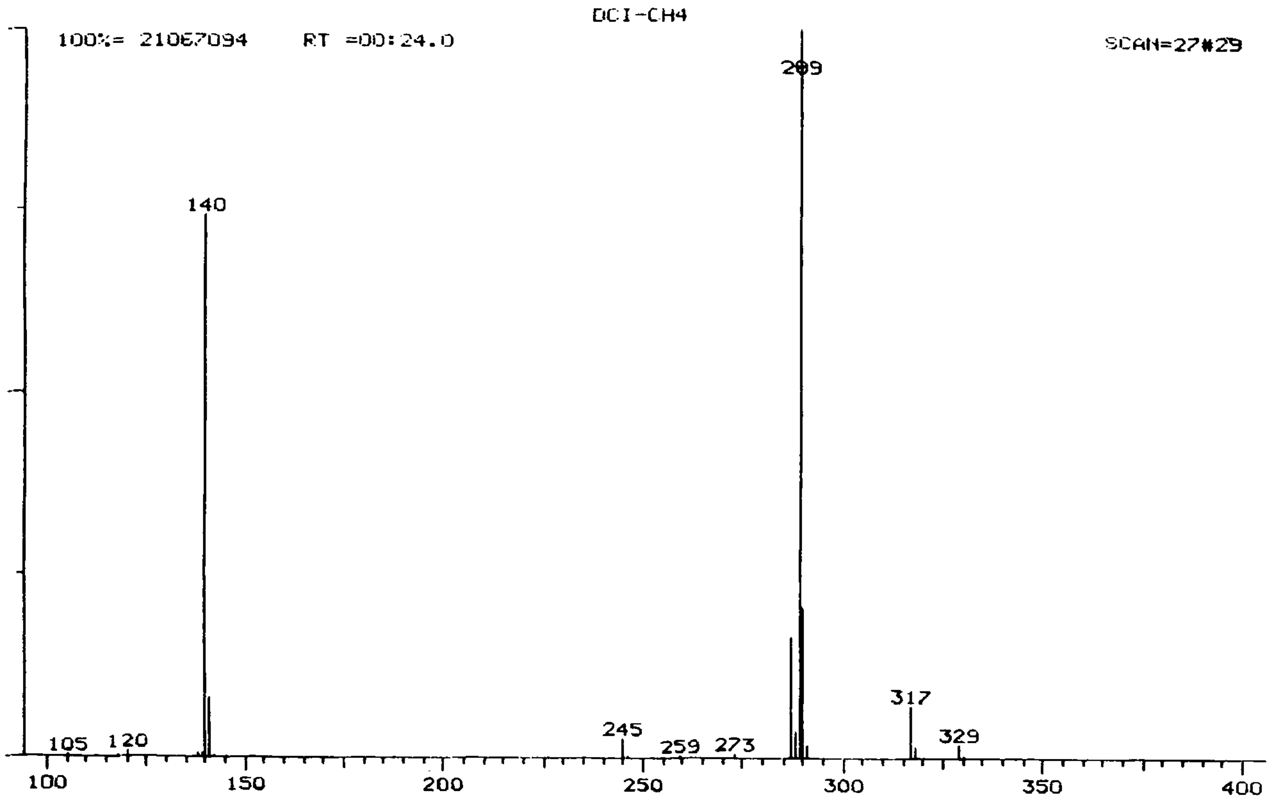


Figure 3. Chemical ionization mass spectrum of bupivacaine.

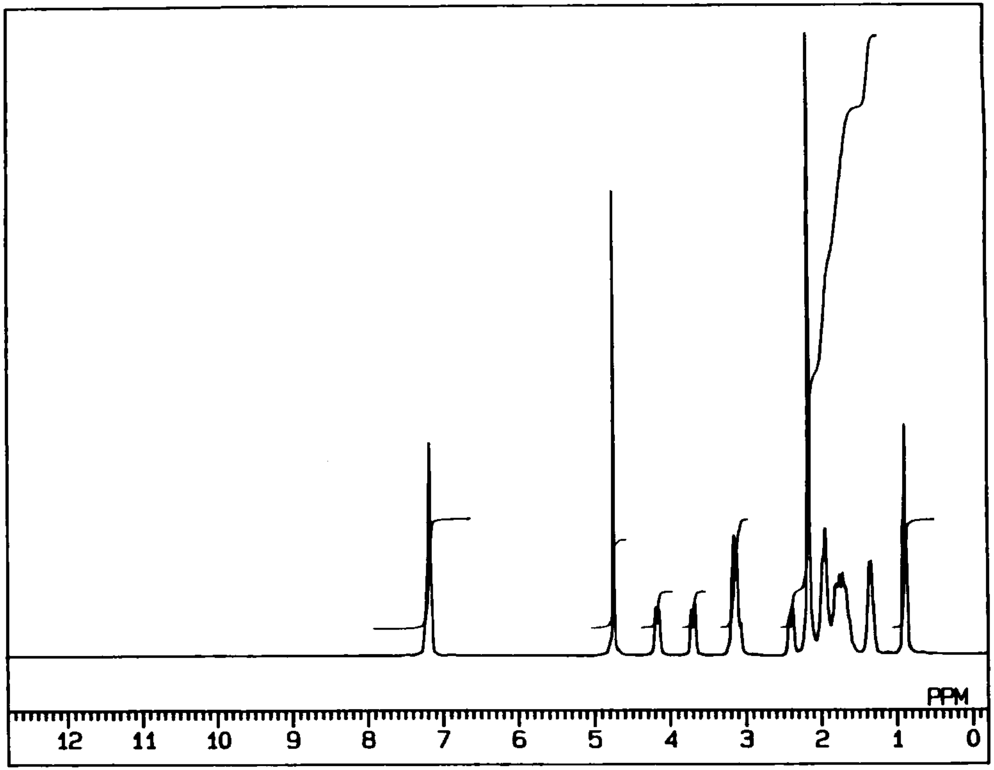
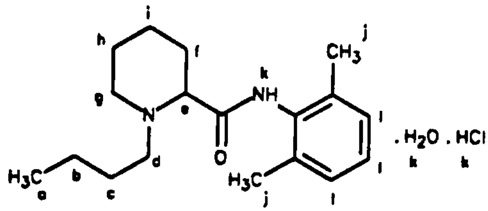


Figure 4. 1H-nuclear magnetic resonance spectrum of bupivacaine.

Table 8



Chemical Shift (ppm, TMS)	No. H	Assignment
0.84	3	CH_3 of the aliphatic chain a
1.20	2	CH_2 adj. to the methyl of the aliphatic chain b
1.5 - 1.8	4	CH_2 * 2 c, i**
1.8 - 2.0	3	CH_2, 1/2 CH_2 of a non equivalent methylene adj. to the methine of the piperidine f, h**
2.13	6	CH_3 * 2 of the phenyl j
2.38	1	1/2 CH_2 of a non equivalent methylene adj. to the methine of the piperidine f
2.99 - 3.21	3	CH_2 of the aliphatic chain adj. to the N of the piperidine and 1/2 CH_2 of a non equivalent methylene adj. to the N on the piperidine d,g
3.65	1	1/2 CH_2 of the non equivalent CH_2 adj. to the N of the piperidine g
4.12	1	CH adj. to the N of the piperidine e
4.72	4	exchangeable protons k
7.0 - 7.2	3	=CH * 3 of the phenyl ring l

**: Individual assignments are uncertain and shift values may be interchanged.

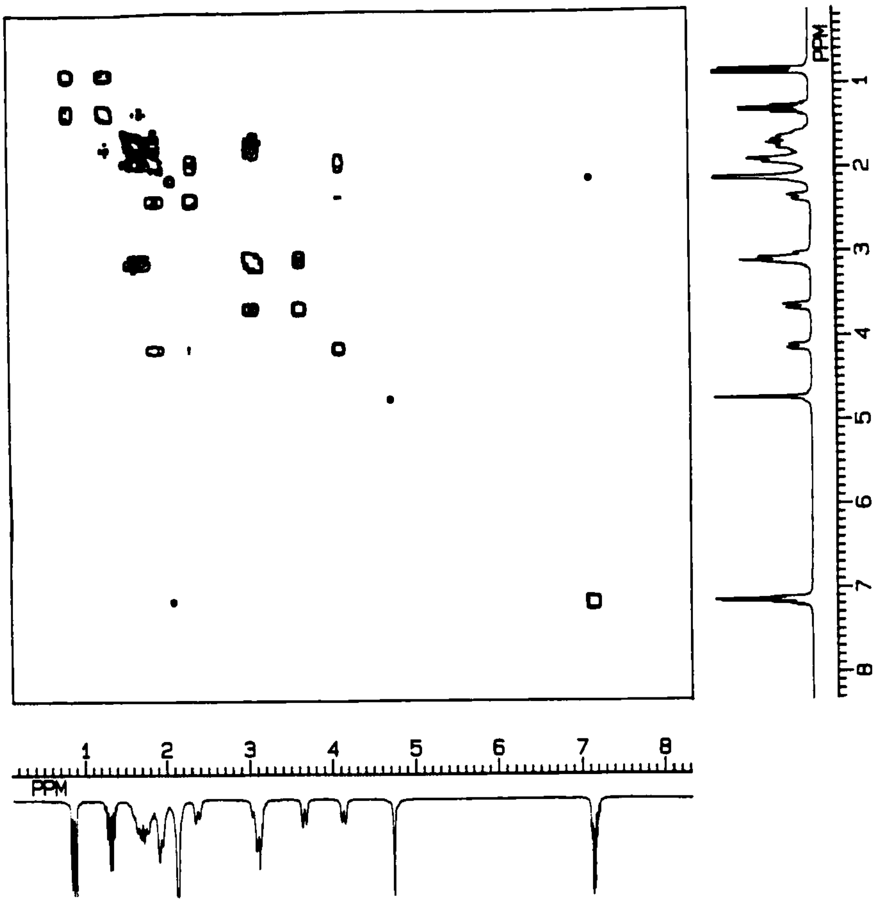
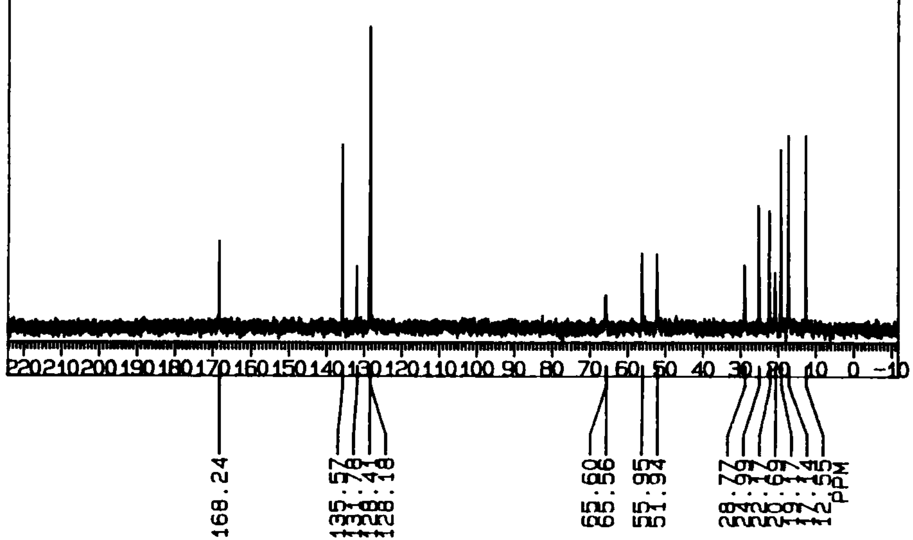


Figure 5. Two dimensional proton correlation spectroscopy (COSY) for bupivacaine.

Figure 6. 13C-nuclear magnetic resonance spectrum of bupivacaine.



instrument as the pmr spectrum. Assignments are listed in Table 9 and were made based on chemical shifts, intensity, distortionless enhancement by polarization transfer (DEPT) experiments and 2-dimensional heteronuclear correlation (HETCOR) experiments. The results from the latter is shown in Figure 7 with the proton spectrum on the y-axis and the carbon spectrum on the x-axis (17).

4.73 <u>Infra-red Spectroscopy</u>

The infra-red absorption spectrum of bupivacaine HCl is shown in Figure 8. It was taken on a Nicolet 20SX FT-IR for a 1% KBr pellet (17).

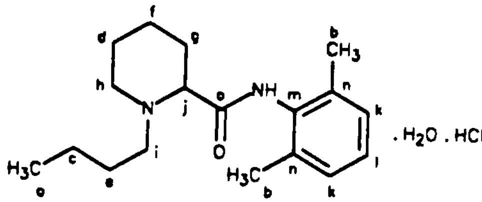
4.74 <u>Ultra-violet Spectroscopy</u>

An ultra-violet spectrum of bupivacaine HCl was obtained on a Varian DMS-200 spectrophotometer. The scan from 300-200 nm on a 0.1 mg/mL solution in water is shown in Figure 9. The wavelength of a maximum absorbance seen is 262 nm, where a molar absorptivity of 473 M^{-1} cm^{-1} was found.

4.8 <u>Dissolution</u>

Official dissolution procedures for bupivacaine have not been established because of the parenteral nature of bupivacaine products. Dissolution methods were described, however, for use with local anesthetic bases and their 3-hydroxy-2-naphthoate salts (15,18). Water jacketed beakers were used with magnetic stirring (360 rpm) using dissolution media of 0.5 M or 0.7 M phosphate buffer at pH 7.4. Withdrawn samples were assayed spectrophotometrically at 263 nm for the bases or 350 nm for the salts.

Table 9



Chemical Shift (ppm, TMS)	Assignment
12.6	a
17.1	b
19.2	c
20.7	*d
22.2	*e
25.0	*f
28.8	g
52.0	h
56.0	i
65.7	j
128.2	k
128.4	l
131.8	m
135.6	n
168.3	o

*: Individual assignments are uncertain and shift values may be interchanged.

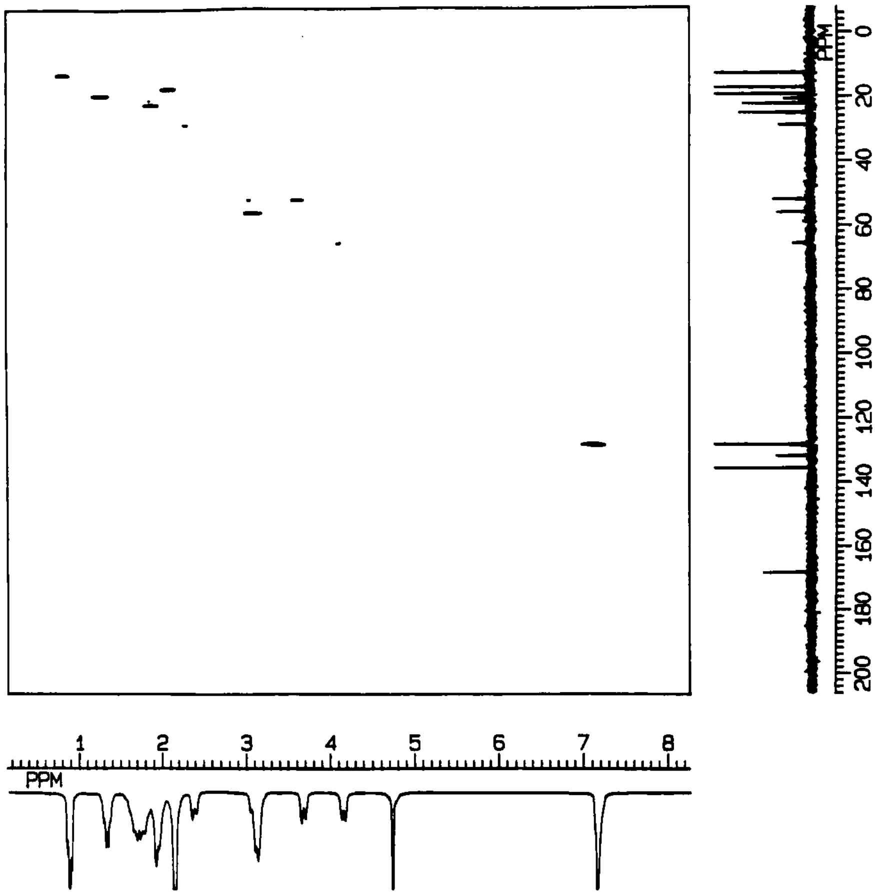


Figure 7. Heteronuclear correlation experiment for bupivacaine.

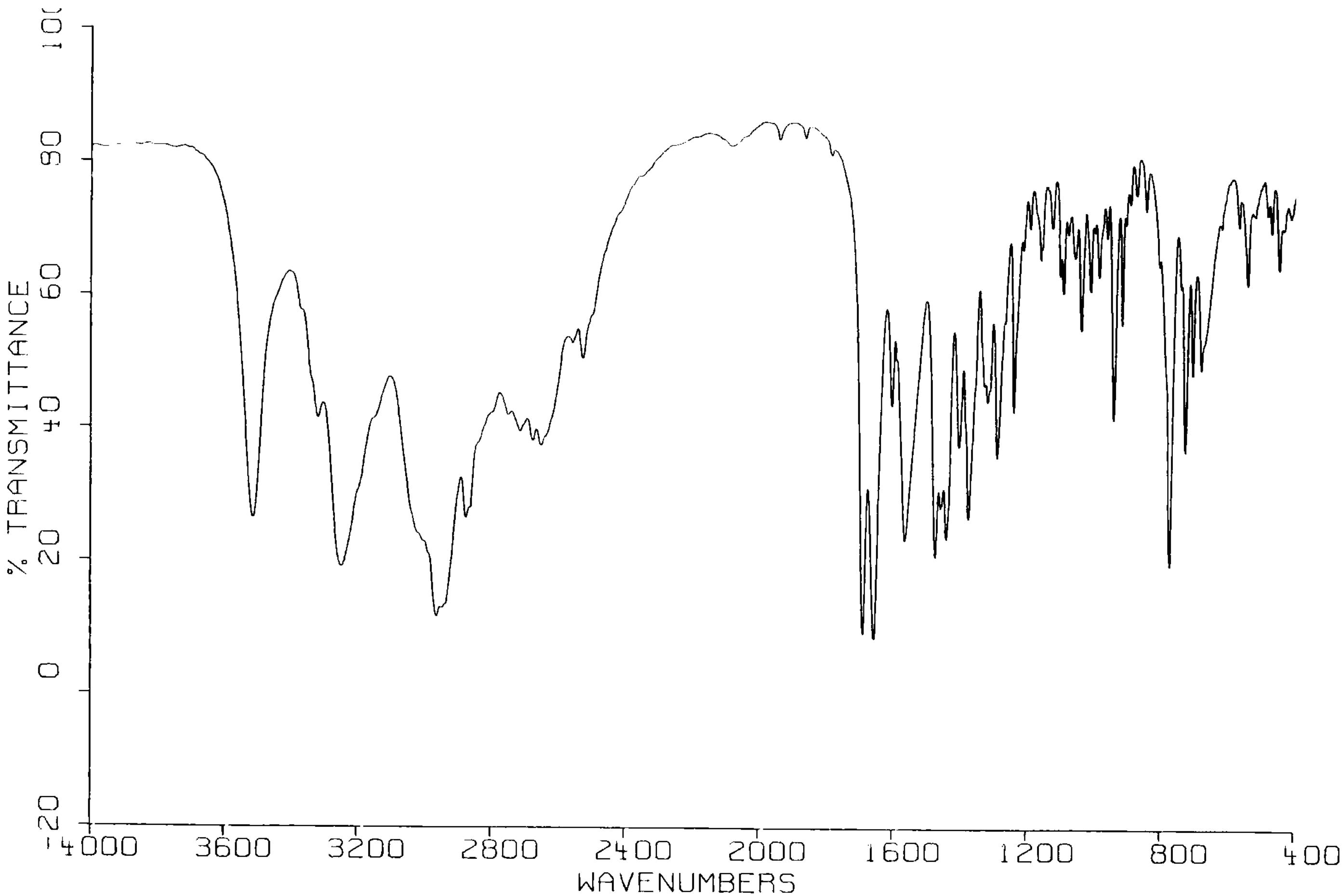


Figure 8. Infra-red spectrum of bupivacaine.

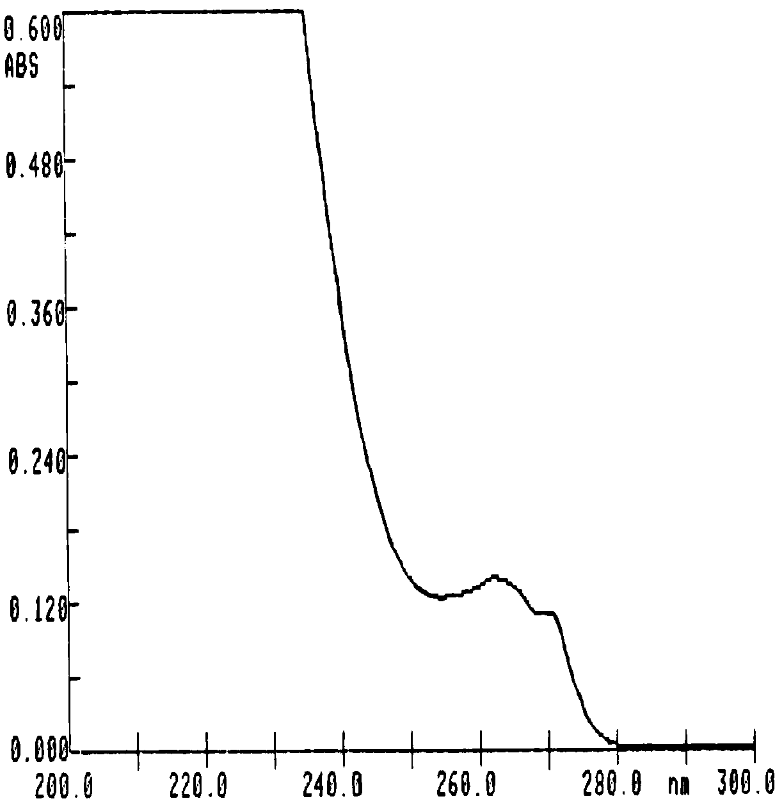


Figure 9. Ultra-violet spectrum of bupivacaine.

4.9 Identification

Bupivacaine hydrochloride drug substance is identified in official compendia by several tests (13,19). An infrared absorption spectrum taken on the extracted and dried base form shows the same maxima as that of a bupivacaine reference standard. Ultraviolet absorption spectroscopy is also used to identify bupivacaine hydrochloride. A 1 in 2000 solution in 0.01 N HCl shows the same absorption maxima and minima as a reference standard plus the absorbance at 271 nm is within 3% of that of the reference standard. The aqueous layer remaining from an alkalinized and extracted solution of the salt also responds positively to the chloride test. Finally, the melting point of a salt formed when trinitrophenol is added to a bupivacaine HCl solution, serves as an identification test.

Identification tests for bupivacaine HCl drug products have also been developed. These include a TLC procedure for Bupivacaine in Dextrose Injection and the organic nitrogenous base infrared absorption spectroscopy test applied to Bupivacaine HCl Injection (3,20).

5. Methods of Analysis

5.1 Titrimetric

Bupivacaine can be determined by a titrimetric procedure in which the compound is dissolved in glacial acetic acid. Crystal violet is used as indicator and the solution is titrated to a green end-point with 0.1 N perchloric acid. Each mL of 0.1 N perchloric acid at this point is equivalent to 32.49 mg bupivacaine HCl (13,19).

5.2 Ultraviolet Spectrophotometry

Ultraviolet spectrophotometry has not been generally applied as a routine or compendial method for bupivacaine analysis although reports have appeared making use of it. These involved dissolution procedures in which the 3-hydroxy-2-naphthoate salt and bupivacaine base were assayed by UV spectrophotometry using 263 nm and 350 nm as analytical wavelengths for the base and salt respectively (15,18).

5.3 Chromatography

5.31 Thin Layer Chromatography

Thin layer chromatography has been used to analyze bupivacaine and has also been used as an identification test and a test for chromatographic purity (13,3,20). It was also used in biological testing to purify extracted rabbit urine (21) and to separate components of extracted equine urine (22). Conditions employed in these procedures are listed in Table 10.

5.32 Gas Chromatography and GC-MS

Gas chromatography is the method of choice for measuring bupivacaine levels in biological fluids in clinical studies. While a multitude of such clinical studies can be found in the literature only a few actually describe chromatographic conditions used. Gas chromatography has also been used for bupivacaine assay in animal studies and in dissolution studies.

The conditions employed in these studies are listed in Table 11. Improvements in methodology indicated in this table include increased use of the more sensitive and specific nitrogen and nitrogen/phosphorus detectors along with the ability to detect metabolites as well as the application of capillary column GC to further increase sensitivity.

Bupivacaine has also been analyzed in biological samples using GC-MS procedures. Conditions for these reported studies are listed in Table 12.

5.33 High Performance Liquid Chromatography

HPLC procedures have been developed for analysis of bupivacaine in both biological specimen and dosage forms. These methods are classified here as achiral or chiral depending on whether or not separation of the (+) and (-) enantiomers was claimed. Conditions used for the achiral methods, all reverse phase, are listed in Table 13.

Table 10
Thin Layer Chromatography of Bupivacaine

Stationary Phase	Mobile Phase	Visualization	R_f	Reference
Silica gel 0.25 mm	butyl alcohol: water:alcohol: gl.HOAc (6:2:1:1)	Iodoplatinate	-	3
Silica gel 0.25 mm	hexane:iso-propylamine (97:3)	Iodine/ H_2SO_4	-	20
Silica gel G	ethanol (96%)	Potassium iodobismuthate	-	13
Silica gel G	isopropanol: chloroform: ammonium hydroxide (10:89.5:0.5)	-	-	21
HP Silica gel 0.2 mm	chloroform: methanol (9:1)	UV/ Degandorff's/ NO_2	0.78	22

Table 11
Gas Chromatography Methods for Bupivacaine

Column	Length	Detector	Col. Temp. (°C)	Gas Flow (mL/min)	Detection Limits (ng/mL)	Percent Recovery	Ref
2.5% SE-30	2 m	FID	210	30	-	-	23
3% OV-17	5'	FID	225	30	-	-	24
3% OV-17	1.8 m	FID	230-280	18	-	-	25
5% OV-17	1.8 m	FID	190-250	23	-	98	26
10% PEG 20M/KOH	1.5 m	FID	214.5	62.2	-	-	18
3% OV-17	1.5 m	FID	265	31.6	20	-	15
3% OV-17	6'	FID	215-235	30	-	-	27
3% SE-30	1.8 m	FID	250	40	50	64	28
10% OV-1 (3% OV-17)	1.2 m (1.8 m)	Nit. Phos.	200-250	35	25	-	29
3% OV-17	1.8 m	Nit. Phos.	180-240	20	50	95.1	30
Carbo-wax20M/ KOH	SCOT 10 m	Nit.	170	5	3	73	31
3% OV-17	6'	Nit.	-	-	10	-	32
5% OV-17	1.8 m	Nit. Phos.	260	30	-	-	33
CPwax 57CB	capil 10 m	Nit.	-	-	1	-	34
3% OV-101	2 m	Nit.	210	30	50	73	35

Nit. - nitrogen detector
Nit. Phos. - nitrogen phosphorous detector

Table 12
GC-MS Analysis of Bupivacaine

Instrument	Column	Electron Energy	Reference
LKB 9000S	-	70 ev	21
Varian 1400gc -MAT311A ms	3% SE-30	88 ev	36
HP 5995A	3% OV-17	70 ev	37
LKB 2091-710	3% JXR	-	38

Table 13

Achiral HPLC Methods for Bupivacaine

Column	Mobile Phase	Detection	Flow Rate (mL/min)	Detection Limit (ng/mL)	Reference
ODS	ACN:28 mM Phos. Buffer pH 6.8 (65:35)	UV-263	2	-	20
C18	MeOH:50 mM Phos. Buffer pH 5.0 (60:40)	UV-254	1	50	39
C18	ACN:50 mM Phos. Buffer pH 5.8 (25:75)	UV-210	0.9	50	40
C8	THF:10 mM Phos. Buffer pH 2.4 (8:92)	UV-210	1.6	100	41
C18	ACN:50 mM Phos. Buffer pH 3.5 (30:70)	UV-210	1	30	42

Chiral separations of bupivacaine optical isomers have been successfully performed using α_1-acid glycoprotein columns originally lab packed since they were unavailable commercially, alone or in combination with other columns. The original commercial column of this type, 'EnantioPac,' was marketed and showed broad applicability to a large number of racemic drug separations although it suffered from a lack of ruggedness. A second generation α_1-AGP column was used in the most recent study listed in Table 14 along with conditions for these separations.

6. Biological Fate and Pharmacokinetics

6.1 Absorption

The absorption, distribution, metabolism and excretion as well as the pharmacokinetics of bupivacaine in man have been extensively reported on and reviewed along with other local anesthetics (12,49,50,51).

Since the drug is available for administration by a variety of routes as indicated in the forward section above, a variety of pharmacokinetic consequences would be expected. Peak concentrations found in venous or arterial plasma samples over all administration routes reported, as summarized by Tucker and Mather, ranged between 0.12 and 4.95 μg/mL with times of peak plasma concentration between 5 and 35 minutes (12). When the data was adjusted to peak maximum concentration in μg/mL per 100 mg bupivacaine dose, however, the range was compressed to 0.2-1.55.

The effect of route of administration on bupivacaine absorption into the systemic circulation can be further delineated by comparing epidural to subarachnoid administration. In the former a two phase absorption has been measured with a rapid initial phase followed by a slower second phase (absorption t1/2 values of 0.12 and 6.0 hours, respectively) (12,51). Following subarachnoid administration, however, no rapid initial absorption was noted (t1/2 = 0.83 hr) while the slower final phase had a t1/2

Table 14
Chiral HPLC Methods for Bupivacaine

Column	Mobile Phase	Detection	Flow Rate (mL/min)	α	Reference
α_1AGP*	50 mM Phos. Buffer, pH 7.01	UV-215	0.5	1.4	43
α_1AGP*	20 mM Phos. Buffer, pH 7.1:2-Propanol (94:6)	UV-215	0.5	1.9	44
α_1AGP**	20 mM Phos. Buffer, pH 7 :2-Propanol (90:10)	-	0.3	1.4	45
α_1AGP**	8 mM Phos. Buffer:2-Propanol (91:9)	UV-215	0.3	1.4	46
α_1AGP***	10 mM Phos. Buffer, pH 7.26:2-Propanol (94:6)	UV-215	-	1.5	47
α_1AGP***	10 mM Phos. Buffer, pH 7:2-Propanol (91:9)	UV-210	0.8	1.3	48

α separation factor (k'2/k'1)
* lab packed column
** first generation commercial column
*** second generation commercial column
α_1AGP alpha$_1$ acid glycoprotein column

of 6.8 hr. These differences have been attributed to differences in administration site vascularity and extent of local binding. In any case the fraction of dose absorbed into systemic circulation from these sites has been measured as 94-96% (51).

6.2 Distribution, Binding and Pharmacokinetics

Following absorption into the systemic circulation bupivacaine is exposed to both tissue and blood binding. High levels of lung binding of bupivacaine have been observed in rat tissue slices (52) whereas circulating blood binding occurs with two plasma proteins; α_1-acid glycoprotein, which contains high-affinity, low-capacity sites and albumin, with low-affinity, high-capacity sites (12). Increases in plasma levels of the former are associated with various disease states and following surgery which can account for higher total blood bupivacaine concentration at that time. Analysis for free bupivacaine, however, showed little increase following cholecystectomy in 7 patients. Prolonged epidural bupivacaine infusion in post operative patients also resulted in high total plasma concentrations but no toxic effects due to protein binding (12). The plasma binding curve for bupivacaine plateaus below 2 μg base per mL plasma at 96% bound (53).

These distribution and binding phenomena are reflected in data shown in Table 15 where the high V_{dss} is related to extensive tissue and plasma protein binding. The clearance and half life values are dependent on site of administration as discussed above with subarachnoid bupivacaine eliminated more rapidly than epidural. Young patients eliminate bupivacaine faster than old patients and the method of calculating these parameters (compartmental or noncompartmental analysis) had little effect on the values obtained.

6.3 Metabolism and Excretion

Bupivacaine and other amide local anesthetics are mainly eliminated through liver metabolism with only 2.6% appearing in the urine unchanged (12,51). Among the metabolic pathways available, Figure 10 indicates those most commonly utilized in mammals including man. Para- and ortho-hydroxylation are

Table 15
Pharmacokinetics Parameters for Bupivacaine

Volume of Distribution Steady State V_{dss} (L)	Clearance (L/min)	Terminal Half Life (hr)	Administr. Route	Reference
73	0.58	2.7	-	12
74	0.45	1.9	IV	33
-	0.51	7.6	epidural/young	54
-	0.33	9.8	epidural/old	54
-	0.46	4.8	spinal/young	34
-	0.28	4.5	spinal/old	34
-	0.61	4.7	subarachnoid	55
67	0.51	2.4	IV/noncompart.	56
68	0.52	2.4	IV/compartment	56
66	0.61	1.8	IV	57

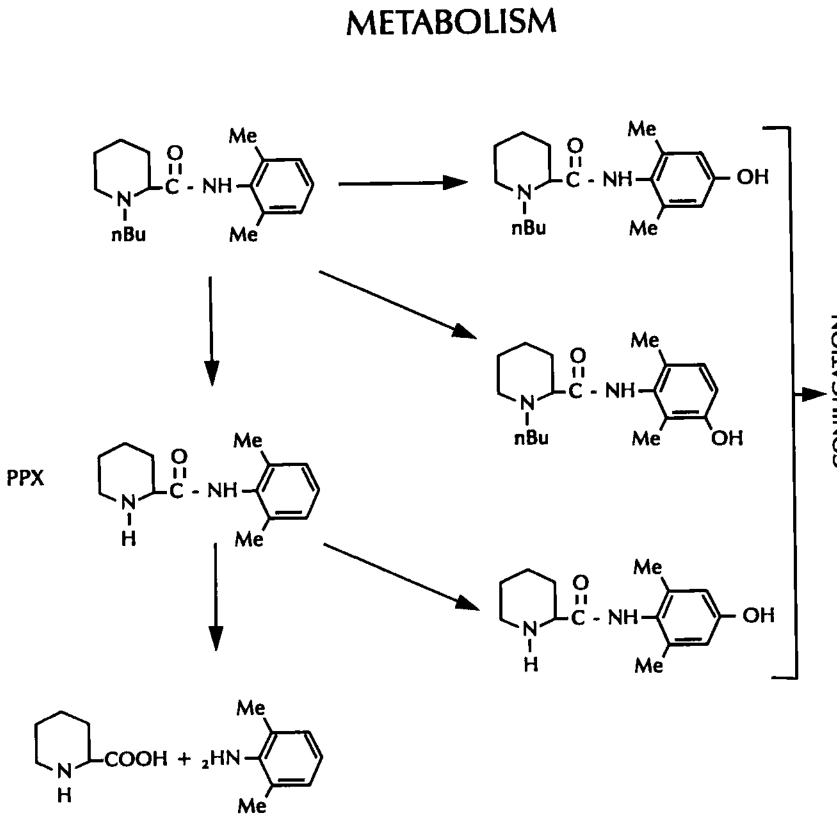


Figure 10. Mammalian metabolic pathways for bupivacaine.

predominant in man, followed by conjugation or N-dealkylation to the intermediate 2,6-pipecolylxylidide (PPX). This can then be p-hydroxylated, and conjugated or hydrolyzed to 2,6-xylidide and pipecolic acid. In man and rats the hydroxylated products predominate with less N-dealkylation and hydrolysis while in monkeys hydrolysis is the major metabolic pathway (12).

7. Determination in Body Fluids

Bupivacaine has been measured in whole blood, plasma, serum, cerebrospinal fluid and urine. The majority of the methods used for these assays were gas chromatographic and gc-ms, with Table 16 listing methods and fluid assayed.

Acknowledgement. The author wishes to thank Ms. N. L. Valcik for manuscript typing assistance, Dr. G. A. Portmann and members of the Molecular Characterization Section of Sterling Research Group - Rensselaer as noted in reference 17.

Table 16
Reference for Bupivacaine Determination in Body Fluids

Method: Fluid	GC	GC/MS	HPLC	TLC
Plasma	35, 24, 31, 28 27, 36, 26, 29 57, 55, 34, 54 33, 58, 59	38, 37, 36 56, 60, 61 62	48, 39, 40, 42	-
Blood	27, 23, 25, 29 59	38	-	-
Serum	30, 32	-	46, 41	-
CSF	24, 29	-	-	-
Urine	25, 58	37, 21, 63 62	41	21

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ANALYTICAL PROFILE OF CEFTAZIDIME

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

(a) 1-[[(6R,7R))-7-(2-Amino-4-thiazolyl)glyoxyl-amido]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]-oct--2-en-3-yl]methyl]pyridinium hydroxide, inner salt, 7[2](Z)-[o-(1-carboxy-1-methyl-ethyl)oxime]. (1,2)

(b) (6R-7R)-7-[(Z)-2-(2-Aminothiazol-4-yl)-2-(2-carboxyprop-2-oxyimino)acetamido]-3-(1-pyridiniummeth-yl)-ceph-3-em-4-carboxylic acid inner salt. (2)

(c) [6R-[6α, 7β(Z)]]-1-[[7-[[(2-amino-4-thiazolyl)[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-2-car-boxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct.-2-ene-3-yl]methyl]pyridinium hydroxide, inner salt. (2)

(d) 1-[[7-[[(2-Amino-4-thiazolyl)[(1-carboxy-1-methylethoxy)imino]acetyl]amino]-2-carboxy-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl] pyridinium hydroxide, inner salt. (1)

1.1.2 Generic Names

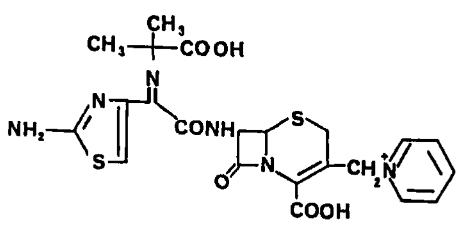
Fortam.

1.2 Formulae

1.2.1 Empirical

$C_{22}H_{22}N_6O_7S_2$

1.2.2 Structural



1.2.3 CAS Registry Number

[72558-82-8] anhydrous
[78439-06-2] penta hydrate.

Ceftazidime pentahydrate 1.16 g is approximately equivalent to 1 g of anhydrous ceftazidime. (3)

1.3 Molecular Weight

546.30 (2)

1.4 Elemental Composition (2)

C 48.33%; H 4.06%; N 15.38%; O 20.50%;

S 11.74%.

1.5 Color and Appearance

White to off white powder.

2. Physical Properties

2.1 Melting Range

Changes to dark brown and decomposes at 135 to 137 °C.

2.2 Solubility

The drug has the solubilities of 5 mg/ml in water and less than 1 mg/ml in alcohol. (4)

2.3 Optical Rotation

$[\alpha]_D^{20} = +24.5°$. (2)

2.4 Action

Ceftazidime has a bactericidal action and broad spectrum activity but increased activity against pseudomonads species (1). Drug is highly stable to hydrolysis by most β-lactamases produced by Gram-negative and Gram-positive bacteria.

2.5 Stability

Drug for injection is essentially stable in the dry state and can be stored at room temperature, but should be protected from light. When reconstituted with H_2O for injections loss of potency occurs slowly and it is recommended that it should be used within 6 hours if stored at room temperature and 24 hours if in refrigerator. (3)

Reconstituted 1 g vials of drug for injection added to 50 ml minibags of sodium chloride injection (0.9%) were found to be stable for 97 days when stored at -20°C. A frozen shelf life of 42 days was suggested, to allow for a refrigeration life of 4 days followed by 24 hours at room temperature. (5)

2.6 pKa

Ceftazidime has pKa's of 1.9, 2.7 and 4.1. (6)

2.7 pH (6)

Ceftazidime sodium solutions have a pH of 5-8 and are light yellow to amber in color depending on the diluent used, concentration of the drug, and length of storage.

2.8 Crystallization of Ceftazidime (7)

Crystals of ceftazidime has been prepared by adjusting an aqueous solution of ceftazidime from pH 5.5-6.5 to 4.0-4.7 at 5-15°C using HCl or H_2PO_4. Thus a suspension of 24 gm of ceftazidime in 5 ml H_2O was cooled to 5°C and adjusted pH 5.9 with 1.28 M NaOH, diluted with H_2O to 150 mg/ml, which was treated with 2.5 M H_3PO_4 at 5°C to pH 4.4 and then 4.2 to give 20 gm of dried crystals.

2.9 X-Ray Powder Diffraction (8)

The X-ray diffraction pattern of ceftazidime was determined on a Philips X-ray diffraction spectrogoniometer fitted with PW 1730 generator. Radiation was provided by a copper target (Cu anode 2000 W, γ = 1.5480 Å) and high intensity x-ray tube operated at 40 KV and 35 MA. The monochromator was a

curved single crystal one (PW 1752/00). Divergence slit and the receiving slit were 1 and 0.1 respectively. The scanning speed of the goniometer (PW 1050/81) used was 0.02-2 θ per second. The instrument is combined with Philips PM 8210 printing recorder with both analogue recorder and digital printer. The goniometer was aligned using silicon sample before use. The x-ray pattern of ceftazidime is presented in Fig. (1). The interplanner distance dÅ and relative intensity I/I_o are shown in Table (1).

2.10 Spectral Properties

2.10.1 Ultraviolet Spectrum (UV)

The ultraviolet absorption spectrum of ceftazidime in 0.1 N H_2SO_4 was obtained on 4054 LKB UV/Vis spectrophotometer (8). The spectrum shown in Fig. (2) exhibited a maximum at 260 nm. The spectra of ceftazidime showed a maximum at 258 nm and 257 nm in H_2O and methanol respectively.

2.10.2 Infrared Spectrum

The infrared absorption spectrum of ceftazidime as KBr was recorded on a Perkin Elmer 580 B Infrared Spectrometer to which an infrared data station is attached (Fig. 3) (8). The structural assignments have been correlated with various frequencies (Table 2).

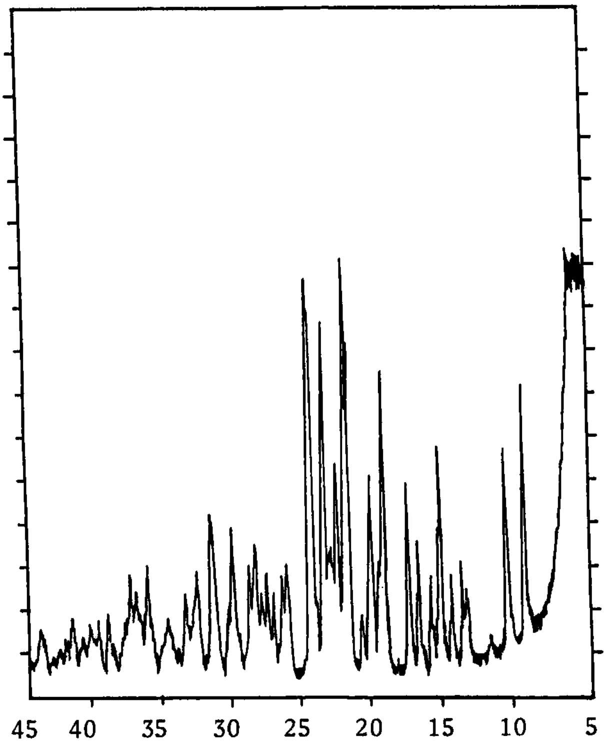


Fig. 1: Characteristic principal lines of the X-Ray powder diffraction of ceftazidime.

Table 1: Characteristic lines of x-ray diffraction of ceftazidime

2 θ	dA	[I/Io %]	2 θ	dA	[I/Io %]
5.496	16.0787	100	36.197	2.4815	15.327
8.815	10.0284	69.011	36.419	2.4669	22.226
10.070	8.7838	53.697	36.882	2.4370	27.451
11.155	7.9318	13.866	37.187	2.4178	17.443
12.877	6.8744	24.115	37.452	2.4012	12.540
13.158	6.7284	29.903	38.422	2.3428	18.488
13.920	6.3616	26.668	39.147	2.3011	15.916
14.770	5.9974	54.541	39.735	2.2682	17.403
15.330	5.7796	27.009	40.294	2.2382	13.504
16.236	5.4591	33.319	40.991	2.2017	17.363
17.037	5.2042	44.855	41.501	2.1759	12.861
17.755	4.9954	8.078	41.966	2.1527	10.450
18.714	4.7415	70.659	42.403	2.1316	9.324
18.952	4.6824	27.009	43.075	2.0999	12.620
19.582	4.5331	49.196	43.468	2.0818	13.625
20.228	4.3898	17.403			
21.296	4.1721	74.236			
21.512	4.1307	97.025			
22.063	4.0288	51.688			
22.533	3.9457	31.993			
22.928	3.8788	82.877			
23.484	3.7881	22.427			
24.073	3.6968	88.424			
25.585	3.4815	29.019			
25.891	3.4411	25.562			
26.590	3.3523	23.553			
27.068	3.2941	27.974			
27.451	3.2490	23.191			
27.907	3.1970	33.480			
28.336	3.1495	26.446			
29.132	3.0653	13.987			
29.584	3.0195	37.138			
29.810	2.9971	15.273			
30.907	2.8931	35.490			
32.123	2.7864	26.688			
32.868	2.7249	24.155			
33.346	2.6869	11.414			
34.181	2.6232	17.805			
34.493	2.6001	10.811			
35.363	2.5381	19.895			
35.613	2.5209	29.099			

I/Io = relative intensity (based on highest intensity as 100)

dA = interplanner distance.

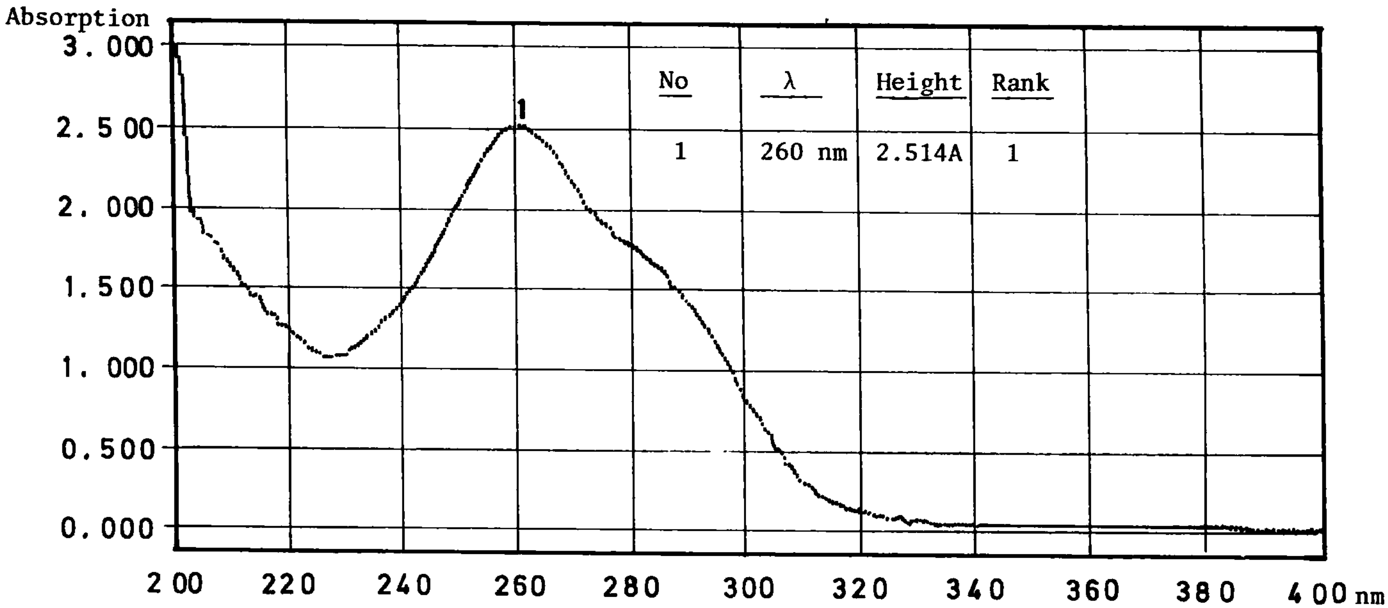


Fig. 2: UV spectrum of ceftazidime in 0.1 N H_2SO_4.

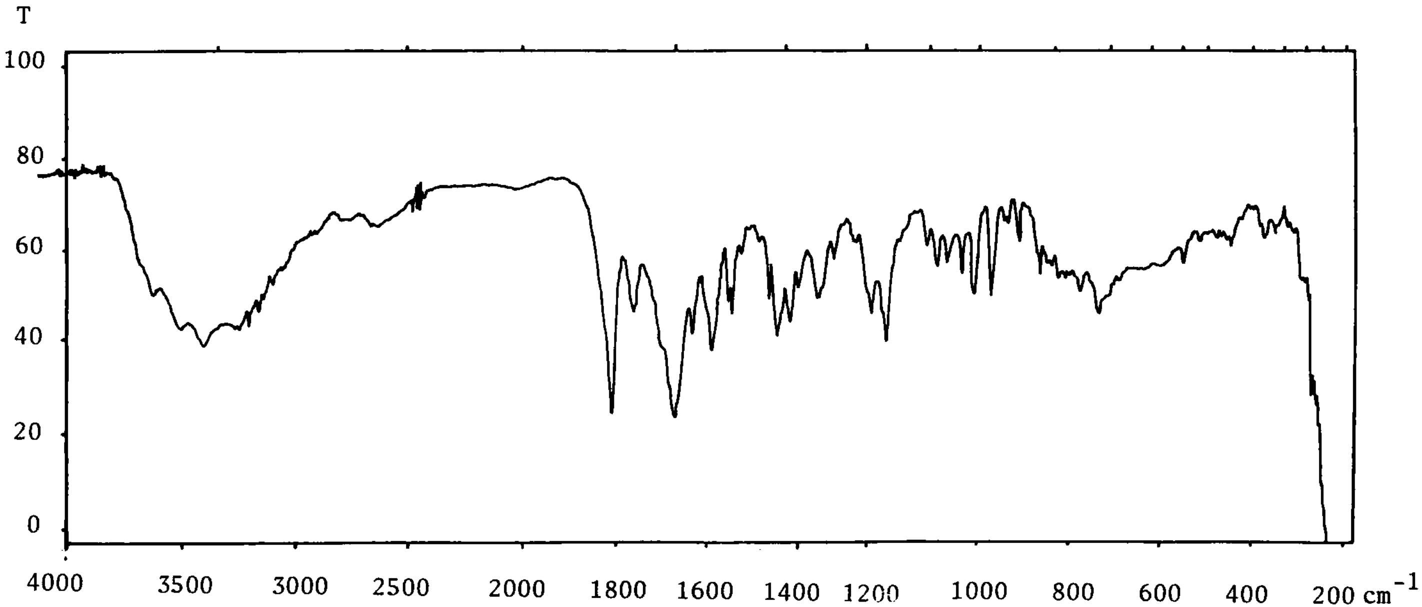


Fig. 3: The IR spectrum of ceftazidime (KBr disc).

Table 2: I.R. characteristics of ceftazidime

Frequency cm^{-1}	Assignment
3000-3600	OH stretching, NH_2, C-H stretch.
1810	C-S-C stretch.
1670	Stretching of amide carbonyl.
1450	C-N
600-800	Adjacent hydrogen deformations.

2.10.3 Nuclear Magnetic Resonance Spectra

2.10.3.1 Proton Spectra (PMR)

The PMR spectra of ceftazidime in DMSO-d_6 was recorded on a Varian FT 80A, 80 MHz NMR spectrometer using TMS as an internal reference. (Fig. 4). The spectral assignments are presented in Table (3).

Table 3: PMR characteristics of ceftazidime

Group	Chemical shift δ (ppm)
C-CH_3	1.38 s
-CH_3	1.70 s
N-CH_2	5.02 d
-NH	6.66 s
-CH_2	8.08 m
-CH_2	9.34 d

s = singlet, d = doublet, m = multiplet.

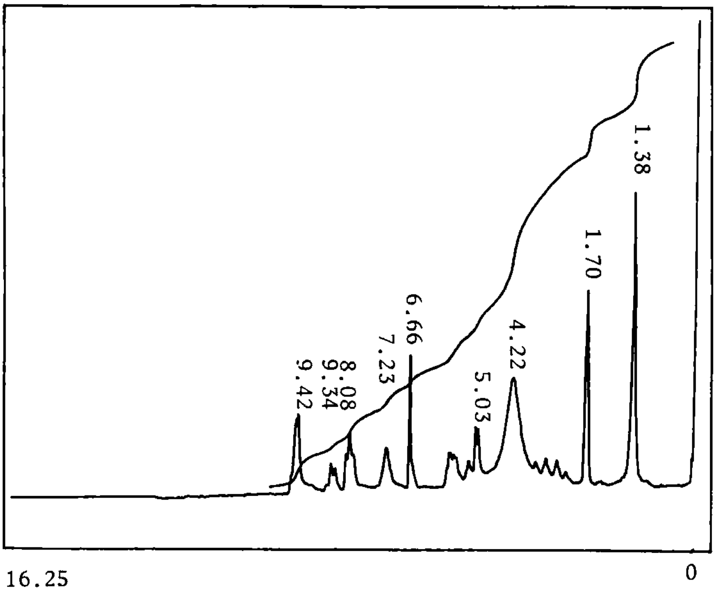


Fig. 4: PMR spectrum of ceftazidime in DMSO-d_6.

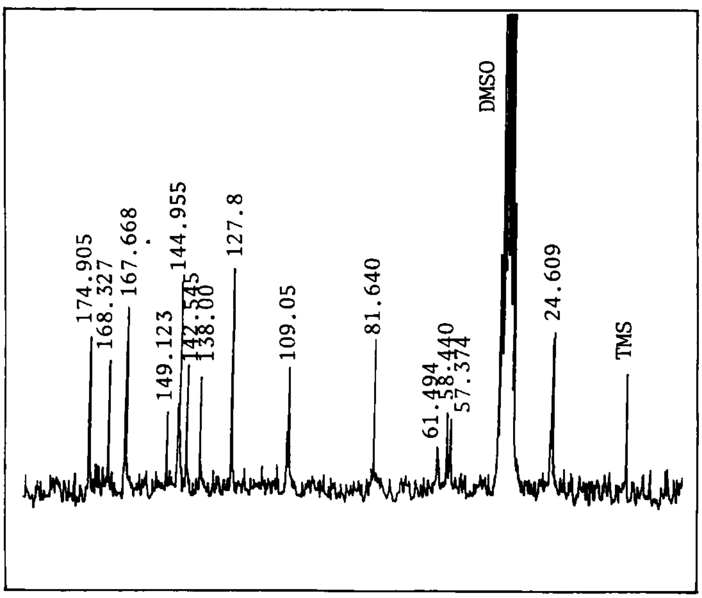


Fig. 5: ^{13}C-NMR complete decoupled spectrum of ceftazidime.

2.10.3.2 ^{13}C-NMR Spectra

The ^{13}C NMR spectrum of ceftazidime in DMSO using TMS as an internal reference is recorded on Jeol FX -100 FT NMR Spectrometer (9) and is presented in Fig. (5).

2.10.4 Mass Spectrum

The mass spectrum of ceftazidime is presented in Fig. (6). This was obtained by electron impact ionization on a Finnigen 300 Mass Spectrometer by direct inlet probe at 270°C. The electron energy was 70 ev. The spectrum was scanned to mass 550 a.m.u. The spectrum shows a molecular ion peak M^+ at m/e 547 with a relative intensity of 40%. The base peak at 169 with a relative intensity of 100%.

3. Synthesis (10)

Ceftazidime is synthesized by the reaction of nitrous acid with ethylacetoacetate to produce oxime. The oxime is next converted to 2-aminothiazole by halogenation with sulfuryl chloride followed by thiourea displacement. The amino group is protected by the trityl amine and then ether formation with ethyl 2-bromo-2-methylpropionate gives intermediate. The sponification then frees the carboxy group for condensation with t.butyl.7-aminocephalosporinate mediated by dicyclohexylcarbodiimide and 1-hydroxy-benzotriazole. The synthesis is completed by removal of the protecting groups from the product formed with trifluoroacetic acid and displacement of the acetoxyl moiety from C-3 by treatment with pyridine and sodium iodide in order to give ceftazidime.

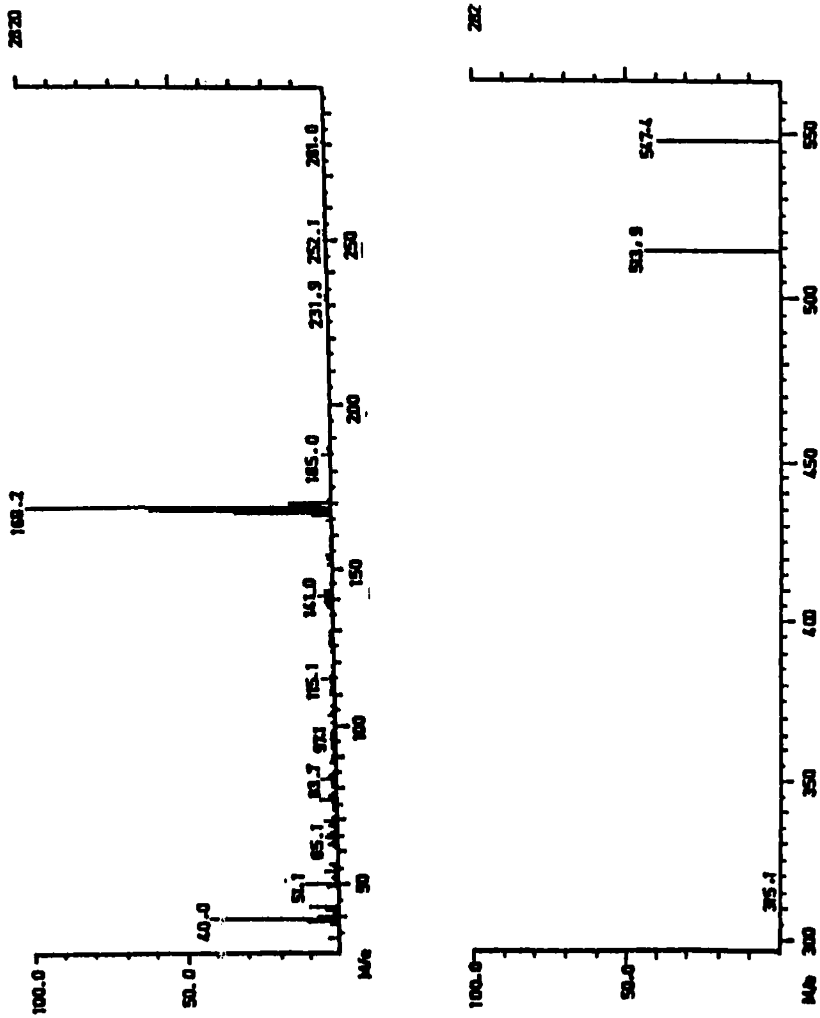
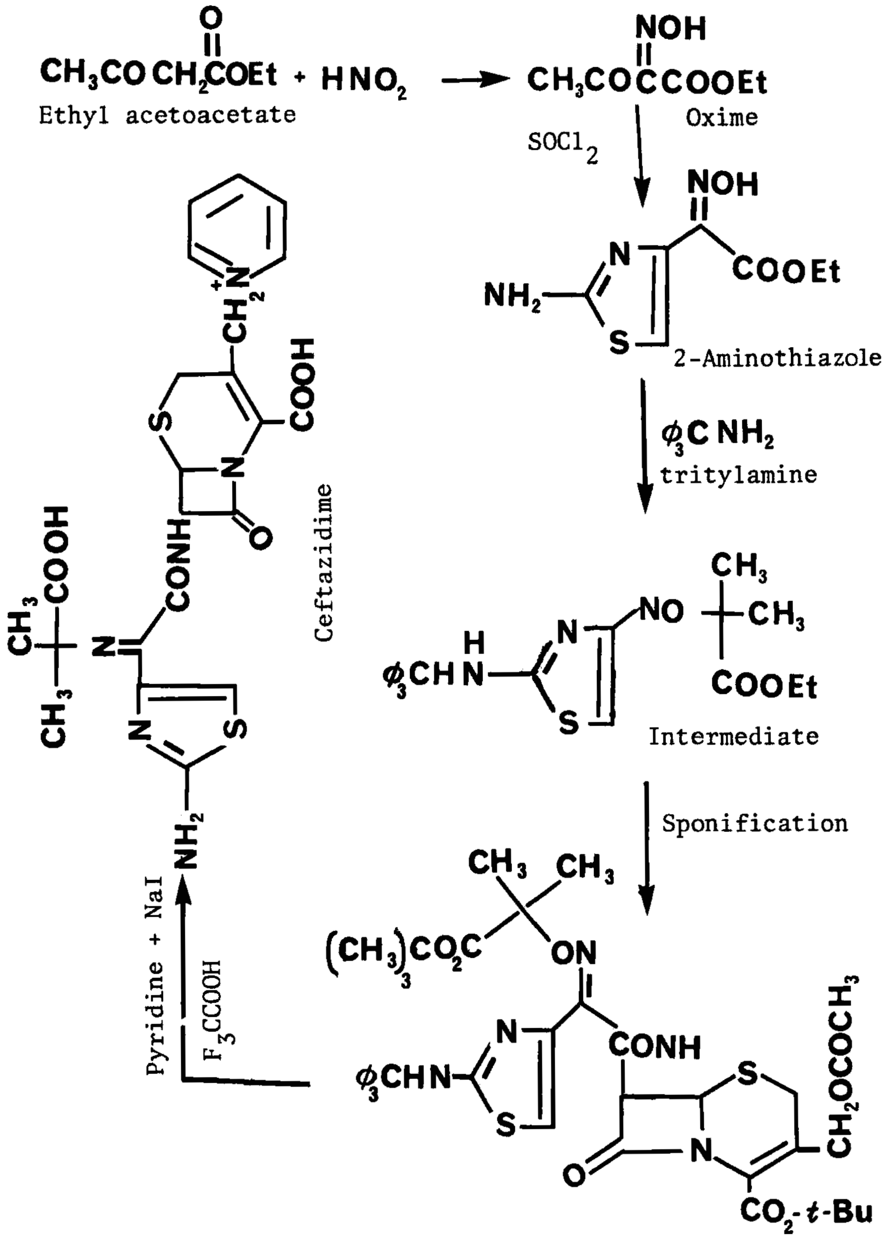


Fig. 6: The mass spectrum of ceftazidime.

Scheme:



Ethyl acetoacetate
Oxime
SOCl2
2-Aminothiazole
tritylamine
Intermediate
Sponification
Ceftazidime
Pyridine + NaI
F3CCOOH

4. Metabolism

Ceftazidime is not absorbed from GI tract and must be given parenterally. IM injection into the gluteus maximus or vastus laterals, ceftazidime may be absorbed more slowly in women than in men. In women, peak serum concentration of the drug may be lower following IM injection into the gluteus maximus than into the vastus laterals. (6)

Ceftazidime is administered by injection as the sodium salt. Mean peak serum concentration of 17 and 39 µg/ml have been reported approximately one hour after intramuscular administration of the equivalent of 0.5 and 1 g of ceftazidime respectively. Five minutes after intravenous bolus injections of the equivalent of 0.5, 1, and 2 gm of ceftazidime, mean serum concentrations of 45, 90 and 170 µg/ml, respectively have been reported. The plasma elimination half life of ceftazidime is about 1.8-2.2 hours but this is prolonged in the patients with severe renal failure and in neonates. It is about 10-17% bound to plasma proteins. (3)

Ceftazidime is widely distributed in body tissues and fluids including bone, synovial fluid, heart, bile, sputum and aqueous humor; therapeutic concentrations have been achieved in the cerebrospinal fluid when the meninges are inflamed, it diffuses across the placenta and is excreted in breast milk. (3)

Plasma concentrations of ceftazidime decline in biphasic manner. In adults with normal renal and heptic function, the distribution half life ($t_{\frac{1}{2}\alpha}$) of ceftazidime is 0.1-0.6 hours and the elimination half life ($t_{\frac{1}{2}\beta}$) is 1.4 to 2 hours. (6)

Ceftazidime is not metabolized and is excreted unchanged principally in urine by glomerular filtration. Following IM or IV administration of a single 0.5 or 1 g dose of drug in adults with normal renal function, 80-90% of the dose is excreted in urine unchanged within 24 hours, approximately 50% of the dose is excreted within 2 hours after the dose. (6)

Serum clearance of ceftazidime average 98-122 ml/min in healthy adults. In geriatic patients 63-83 years of age with urinary tract infections, serum clearance of ceftazidime averaged 79 ml/min and the serum half life of a drug averaged 2.9 hours. In patients with cystic fibrosis, the serum clearance of ceftazidime ranges from 142-316 ml/min per 1.73 M^2, the serum half life of the drug in these patients, however, ranges from 1-2.2 hours and generally within the same range as that for healthy individuals. (6)

The serum half life of drug is longer in neonates than in older children and adults. Serum concentration of drug are higher and the serum half life of drug is prolonged in patients with impaired renal function. The serum half life of the drug; only slightly prolonged in patients with impaired hepatic function and accumulation of the drug does not generally occur in those patients unless renal function is also impaired.

Ceftazidime is readily removed by hemodialysis. The drug is also removed by peritoneal dialysis. (6)

5. <u>Uses and Requirements</u>

Ceftazidime is third-generation cephalosporin antibiotic with enhanced activity against Pseudomonas aeruginosa. It is used in the treatment of susceptible infections including respiratory-tract infections such as pneumonia and lung infections in patients with cystic fibrosis-urinary tract infections, skin and soft tissue infections, bone and joint infections, peritonitis and other abdominal infections, septicaemia and meningitis. It is often used alone but can be used in association with an aminoglycoside or vancomycin in patients with severe neutropenia, or if infection with Bacteraides fragilis is suspected, it may be used in association with an antibiotic active against anaerobes such as clindamycin or metronidazole. The drug should generally be administered separately. (3)

Ceftazidime is used for the treatment of gynecologic infections (including endometritis, pelvic cellulitis, and other infections of female genital tract). (6)

Ceftazidime is available as the pentahydrate but it is formulated with sodium carbonate to form the sodium salt in solution. Doses are expressed in terms of anhydrous ceftazidime. It is administered by deep intramuscular injection, slow intravenous injection, or intravenous infusion in doses of 1-6 g daily in divided doses every 8-12 hours. The higher doses are used in severe infections especially in immuno-compromised patients. In patients with cystic fibrosis who have pseudomonal lung infections, high doses of 100-150 mg per kg body weight daily in 3 divided doses upto 9 g daily has been given to adults with normal renal function . If pain is considered a problem with IV use, drug may be reconstituted with lignocaine hydrochloride 0.5% or 1% injection. (3)

30-100 mg per kg in 2 or 3 divided doses of drug are usually given to the children and may increase upto 150 mg per kg daily to a maximum of 6 g daily may be given in 3 divided doses.

Neonates and infants upto 2 months old have been given 25-60 mg per kg daily in 2 divided doses. (3)

6. Cautions and Adverse Effects

Adverse effects reported with ceftazidime are similar to those reported with other cephalosporins. Ceftazidime is generally well tolerated, adverse effects have been reported in about 9% of patients receiving the drug and have been required discontinuance in about 2% of patients. (6)

Eosinophilia, thrombocytosis, transient leukopenia, neutopenia, thrombocytopenia and lymphocytosis may occur in 2-7% of the patients receiving ceftazidime. (6)

Adverse GI effects, including diarrhea, nausea, vomiting, abdominal pain and a metallic taste have been reported in 2% of the patients. (6)

Rash (maculopapular or erythematous) puritus, urticaria, fever and adverse local reactions including (phlebitis and pain or inflammation at the infection site have been reported in less than 3% of the patients receiving ceftazidime.

Transient increase in serum concentrations of SGOT, SGpt, Alkaline phosphatase, LDH and/γ-glutamyl-transferase (γ-glutamyl transpeptidase, GGT, GGTP), bilirubin, bun and serum creatinine have been reported in 1-9% of the patients, receiving ceftazidime in appropriately large doses, may cause seizures, especially in patients with renal impairment. The drug should be discontinued promptly if seizures occur; anticonvulsant therapy may be administered if indicated. If acute overdosage of ceftazidime occurs, hemodialysis may be used to enhanced elimination of the drug. (6)

It has been reported that ceftazidime does not cause decreased activity when incubated in solution with gentamycin or tobramycin at 37°C (11,12). However, the manufacturers recommended that ceftazidime, like most other β-lactam antibiotics, should not be mixed with an aminoglycoside in the same giving set or syringe because of potential interaction. Cairns and Robertson (13) reported the physical incompatibility between ceftazidime and vancomycin.

7. Methods of Analysis

7.1 Elemental Analysis

The elemental analysis of ceftazidime as reported (2) is:

$C_{22}H_{22}N_6O_7S_2$ = 546.30

Element	% Theoretical
C	48.33%
H	4.06%
N	15.38%
O	20.50%
S	11.74%

7.2 Colorimetric Method (14)

A colorimetric method for the determination of ceftazidime has been developed which is based on

formation of diazo products with $NaNO_2$ in acidic medium. The color (absorbance) was measured at 500 nm. A linear relation between the color intensity and drug content was observed in the concentration range 12.5-200 µg/ml.

7.3 Microbiological Methods

(a) A sensitive method (15) is described for the determination of ceftazidime in biological fluids using Proteus morganii NCTC235 as the test organism. The organism is cultured overnight on nutrient agar slopes at 37°, the growth is then washed off in 5 ml phosphate buffer at pH 7.0, and the suspension is diluted 10-fold to contain about 10^7 cells/ml and added in an amount equal to 1% to the molten agar held in Petri dishes at 50°. About 12.5 ml of inoculated medium is poured into 9-cm plates (large 12 inches plates can also be used). Holes are punched in the agar for standard and test solutions. After 18 hours incubation at 37°C, zone dimension are measured and ceftazidime concentrations in the samples are estimated from a standard curve.

(b) Michael et al. (16) analysed ceftazidime in serum and body fluids by large-plate agar diffusion technique using standard solutions of the drug made up in human serum. Serum samples were assayed against standards made up in 100% serum and each test serum sample and standard (25 µl) was inoculated into Whatman AA paper disks. Standard solutions for assaying blister fluid and cantharidin blister fluid were prepared with 50 and 70% serum respectively made up in phosphate buffer (pH 7). Blister fluid aspirates and standard (5 µl) were inoculated onto 6-mm disk punched from Whatman no. 54 filter paper. Cotton threads were removed and immediately transferred into preweighed bottles which were then weighed. The thread lengths were measured, and the amount of fluid taken up per centimeter of length was calculated. The threads were then cut into two 1-cm lengths for assay. Standards for the threads were prepared by impregnating 1-cm lengths of dry thread with 5 µl of known conc. of antibiotic dissolved in 50% serum.

Disks impregnated with serum and blister fluids were plated into the unpunched surface of large assay plates. The threads were inserted into 3-mm diameter wells cut into agar plates, and 5 µl of saline was added to each well to assist uniform elution of the antibiotic. Serum conc. in excess of 50 µg/ml were assayed by using Bacillus subtilis 1904 seeded into Oxoid Antibiotic assay medium No. 2 with 3 g of sodium citrate added per litre. Concs of drug in blister and thread fluids and serum concentrations of less than 50 µg/litre were assayed by using proteus morganii 235. In these assays the plates consisted of a base layer of nutrient agar and a top layer of Oxoid Isosensitest agar into which the organism was seeded.

All assay plates were incubated overnight at 37 °C. For the threads, the assay results were corrected for the volume of fluid absorbed (4 to 5 µl/cm) before the antibiotic concentrations were calculated

7.4 Chromatographic Methods

High Performance Liquid Chromatography

Some of the rapid and sensitive high-pressure liquid chromatography (HPLC) procedures for the quantitative analysis of ceftazidime are summarised in Table (4).

7.5 Thermal Behavior

A differential scanning colorimetry curve was obtained Fig (7) on a Perkin-Elmer DSC-2C differential calorimeter. Nitrogen was used as the purge gas. Scan was performed at the rate of 25 °C/min from 60°-380 °C. The DSC curve revealed an endothermic melting peak (Max. 132.71 °C).

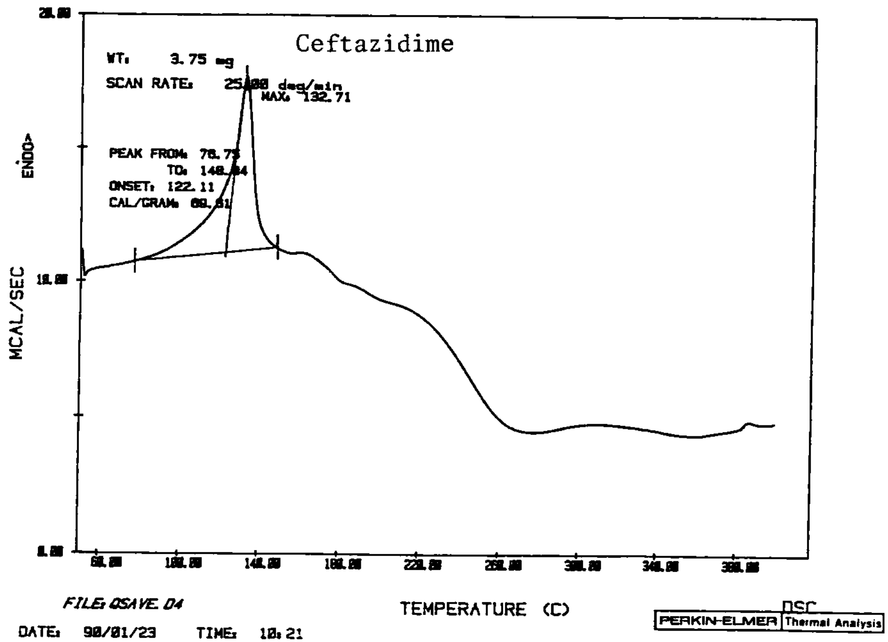


Fig. 7: The differentical scanning calorimetric (DSC) thermal curve of ceftazidime.

Table 4: Summary of HPLC conditions for the determination of ceftazidime.

Column	Mobile phase	Flow rate	Sample	Detection	Ref.
(30 cm X 3.9 mm of μ Bondapak C_{18} (10 μm).	Acetic acid:H_2O: acetonitrile, pH 4.0.	2 ml/min	Serum, urine CSF or peri- tonial fluid.	254 nm.	17
(30 cm X 3.9 mm) of μ Bondapak C_{18} (10 μm) and a guard column of μ Bondpak C_{18} of (37 to 50 μm).	Phosphate buffer (pH 6.5): methanol 41:9 for serum 22:3 for urine.	1.2 ml/min for serum 2.0 ml/min for urine.	Serum or urine.	255 nm	18
(10 cm X 5 mm) of Hypersil ODS (μm).	0.05 M-$NH_4H_2PO_4$ containing 7% of acetonitrile and 0.1% of formic acid.	1 ml/min.	Serum or plasma	--	19

Continued /...

Continued (Table 4) ...

Column	Mobile phase	Flow rate	Sample	Detection	Ref.
Micropak MCH10	20% methanol in 50 mM-$NH_4H_2PO_4$ that in 117 µm in $HClO_4$.	1 ml/min.	Blood or urine	257 nm	20

8. References

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DICLOFENAC SODIUM

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1 Description

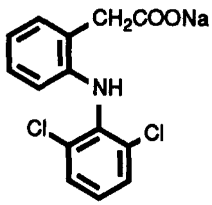
Synonyms: Voltaren, Voltarol, Voldal, Voveran, Orthophen

1.1 Introduction

Diclofenac sodium is a synthetic, nonsteroidal anti-inflammatory, and analgesic compound

1.2 Formula, Name, Formula Weight

Formula



Diclofenac Sodium

Formula Weight 318.13

$C_{14}H_{10}Cl_2NO_2Na$

Diclofenac sodium is also described by the following chemical names:

1) 2-[(2,6-Dichlorophenyl)amino] benzeneacetic acid monosodium salt
2) [0-(2,6-dichloroanilino)phenyl] acetic acid sodium salt
3) sodium[0-[(2,6-dichlorophenyl)amino]phenyl] acetate

1:3 Appearance, Color, Odor

Diclofenac sodium is an odorless, white to off-white crystalline, slightly hygroscopic powder.

2 Physical Properties

2.1 Ultraviolet Spectroscopy

The ultraviolet spectra of diclofenac sodium (batch 46694), in two solvents, methanol and phosphate buffer pH 7.2, were obtained with IBM UV/visible 9420/9430 spectrophotometer in our laboratory. The spectra depicted characteristic aromatic absorption (Figure 1). The wavelengths of maximum absorption for the two solvents are 283 and 276 nm and the molar absorptivities ε_{283} and ε_{276}, are 1.05 x 10^5 and 1.01 x 10^5 liter/mole-cm respectively.

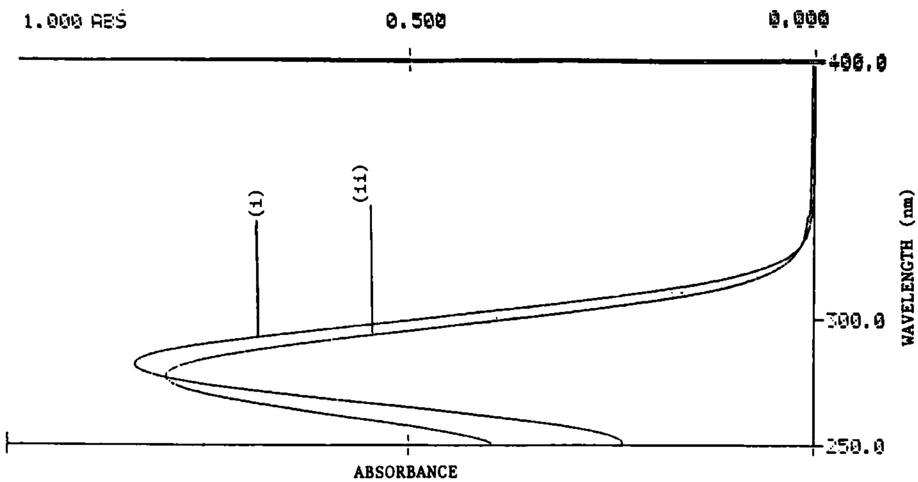


Figure 1 Ultraviolet Absorption Spectra of diclofenac sodium in methanol (i). and aqueous phosphate buffer (pH 7.2), (ii). b=1cm c = 7.86 x 10^{-6}M

2.2 Mass spectrum

The mass spectrum of diclofenac sodium was obtained with a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer and a Shimadzu 9060s multiple-ion-detector peak matcher were used. Diclofenac and 4'-methoxydiclofenac (internal standard) were first converted to their respective indolinone derivatives. The electron-impact mass spectra (20eV) of indolinone derivatives of diclofenac and 4'-methoxydiclofenac are shown in Figure 2 (1).

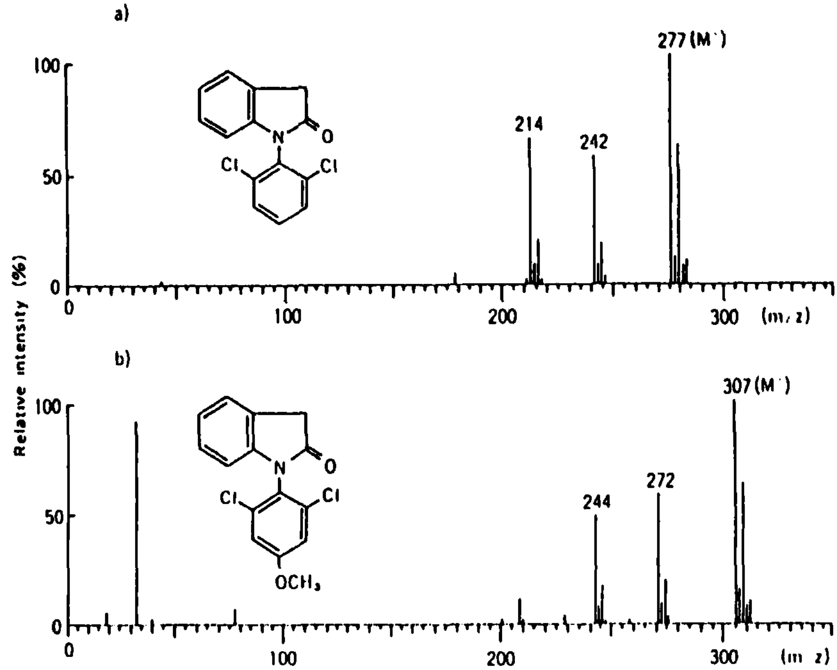


Figure 2 Electron - Impact mass spectra (20eV) of indolinone derivatives of a). diclofenac; b). 4'-methoxydiclofenac

2.3 Nitrogen-15 Nuclear Magnetic Resonance Spectrum

The ^{15}N NMR of diclofenac sodium was measured in 20% solution of hexadeuteriodimethyl sulfoxide (DMSO-d_6). The chemical shift of ^{15}N of diclofenac sodium was -294.4 (2).

2.4 Nuclear Magnetic Resonance Spectrum

The ^1H NMR of diclofenac sodium was recorded in deuterated methanol(d_4) with sodium acetate as internal standard and using a Varian T60-A NMR spectrometer (3).The spectral assignments are presented in Table 1.

Table 1

^1H - NMR Assignment for Diclofenac Sodium

Chemical shift δ (ppm)	Multiplicity	no. of atoms	Assignment
3.62	singlet	2	$-CH_2-$
6 -7.5	multiplet	7	aromatic protons

We also recorded the ^1H NMR spectrum of diclofenac sodium with an IBM AF/250 spectrometer in the pulse mode using deuterated methanol as the solvent. The spectral assignments is similar to the spectrum reported by Fattah et al. The methylene protons appears as singlet with a chemical shift of 3.53. The chemical shifts of the aromatic protons are 6.24-7.28 and exhibits as multiplet. The spectrum is presented in Figure 3.

2.5 Crystal properties

The crystal strucutre of the tetrahydrate of diclofenac sodium was determined by Reck et al. The compound crystallized in the monoclinic space group $p2_1/m$ with a=9.464(2), b=39.405(7), c=9.972(3) Å and β=90.72(2)°. The unit cell contained two symmetry independent formula units $(C_{14}H_{10}Cl_2NO_2)^-Na^+.3.94\ H_2O$ (4).

2.6 Differential Scanning Calorimetry

The differential scanning calorimetry (DSC) of diclofenac sodium was done with a Dupont 1090B thermal anlyzer at 20°C/min in static air (Figure 4). The analysis shows an exotherm at 280° C,followed by an endotherm. This result is indicative of melting and decomposition. The normal melting point range of the drug is 283-285° C.

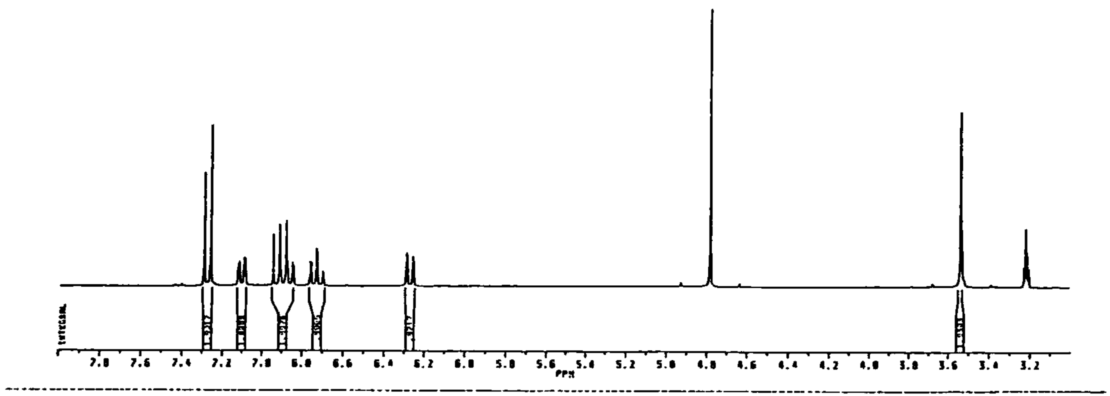


Figure 3. ^{1}H NMR of diclofenac sodium

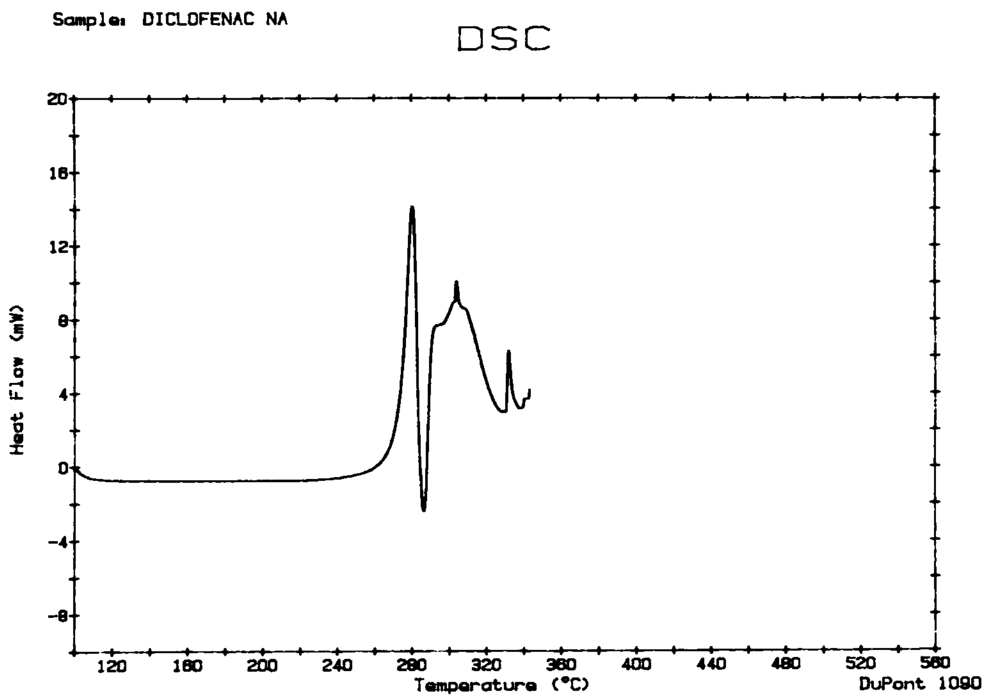


Figure 4 DSC curve of Diclofenac sodium

2.7 X -Ray Diffraction

X -Ray structure analysis of diclofenac sodium showed that the two aromatic rings were twisted in relation to each other, the angle of torsion being 69° and the hydrogen bond $\delta = 2\times10^{-1}$ nm. The two chlorine atoms substituted into the phenyl ring are responsible for the maximum twisting of the ring. The relative steric arrangement of the aromatic rings is known to influence receptor interaction of some non-steroidal anti-inflammatory drugs including diclofenac (5,6).

2.8 Solubility:

The equilibrium solubility performed in various solvents at the indicated temperature (RT) are shown in Table II.

Table II

Solubility of Dicolfenac Sodium

Solvent	Temperature	Solubility (mg/ml)
Deionized Water (pH 5.2)	RT	>9
Methanol	RT	> 24
Acetone	RT	6
Acetonitrile	RT	< 1
Cyclohexane	RT	< 1
pH 1.1 (HCl)	RT	< 1
pH 7.2 phosphate buffer	RT	6

2.9 Dissolution Constant (pka) and Partition Coefficient

The pka of diclofenac sodium in water is 4 and the partition coefficient in n-octanol/aqueous buffer pH is 13.4 (7).

3 Synthesis

3.1 Synthesis of Diclofenac Sodium

Synthetic procedures of diclofenac sodium have been described (8-12). The synthetic pathway by Tamura *et al* is presented in Figure 5 (8). The α-(methylthio)acetanilide 2 was obtained by N-acylation of N-phenyl-2,6- dichloroaniline 1. The product 2 was oxidized with m-chloroperbenzoic acid (m-CPBA) or hydrogen peroxide (H_2O_2) to give α-(methylsulfinyl) acetanilide 3. Cyclization of 3 to form product 5 was carried out by heating 3 in benzene with p-toluenesulfonic acid (pTsOH). An alternate route through chlorination of 2 with N-chlorosuccinimide (NCS) in carbon tetrachloride (CCl_4) gave 4 and subsequent cyclization of 4 with stannic chloride ($SnCl_4$) yielded 5. Desulfurization of 5 with Raney Nickel (Ra-Ni) or Zinc dust - acetic acid (Zn - AcOH) obtained the oxindole 6. Hydrolysis of 6 with sodium hydroxide gave diclofenac sodium 7.

3.2 Synthesis of [^{14}C] Diclofenac Sodium

The synthesis of ^{14}C labelled diclofenac sodium was achieved in 22% overall yield (13). The synthetic scheme is presented in Figure 6. It began with the preparation of ^{14}C labelled (o-iodophenyl) acetonitrile 2 by the substitution of (o-chloromethyl)iodobenzene 1 with [^{14}C]KCN in mixture of 18 crown-6 in acetonitrile (14). The cyano group of 2 was converted to carboxylic acid 3 through acid hydrolysis in 18N H_2SO_4 . Esterification of the acid in MeOH-CH_2Cl_2 afforded the methyl ester 4. Coupling of the ester with 2,4-dichloroaniline using cuprous iodide (CuI)-potassium carbonate (K_2CO_3) yielded the diphenylamine 5. Base hydrolysis of the ester group of 5 to acid 6, followed by reacting the acid with equimolar of sodium hydroxide in water obtained the final product [^{14}C] diclofenac sodium 7.

4 Stability

Diclofenac sodium tablets film coated with polymers like acrylate hydroxypropylcellulose were reported to be stable after storage for one week at 30°C in 80% relative humidity (15). Suppository formulation was also analyzed for stability using thin layer chromatography and ultraviolet spectroscopy. The formulation was stable for 24 months at room temperature (16). Stability in biological fluid (serum) was determined and the results demonstrated that diclofenac sodium can be frozen for at least two weeks without degradation (17).

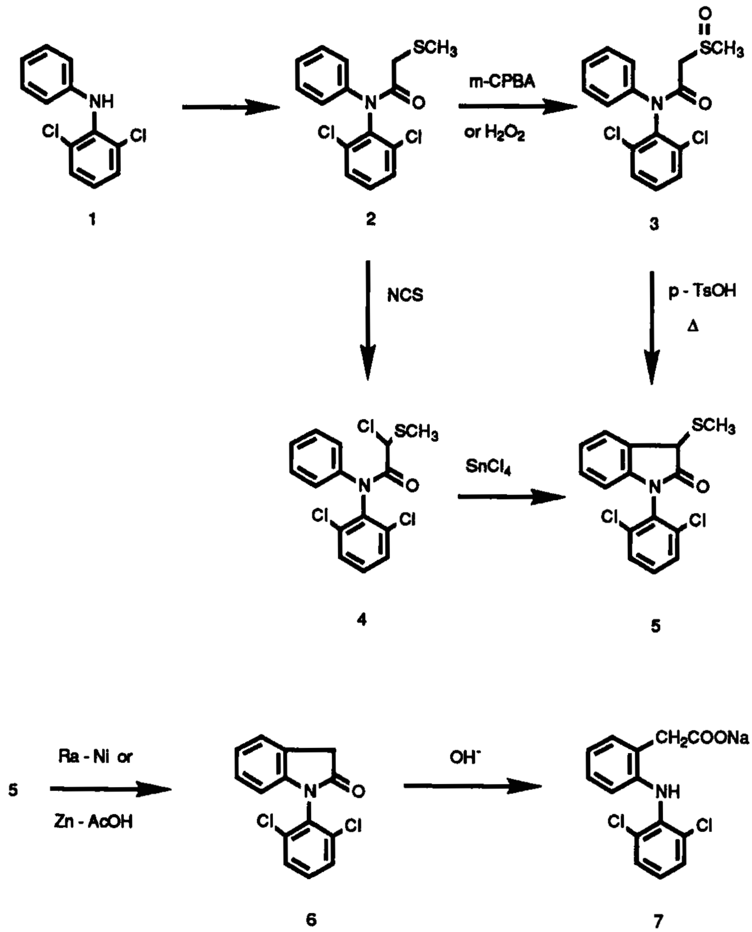


Figure 5. Synthesis of diclofenac sodium

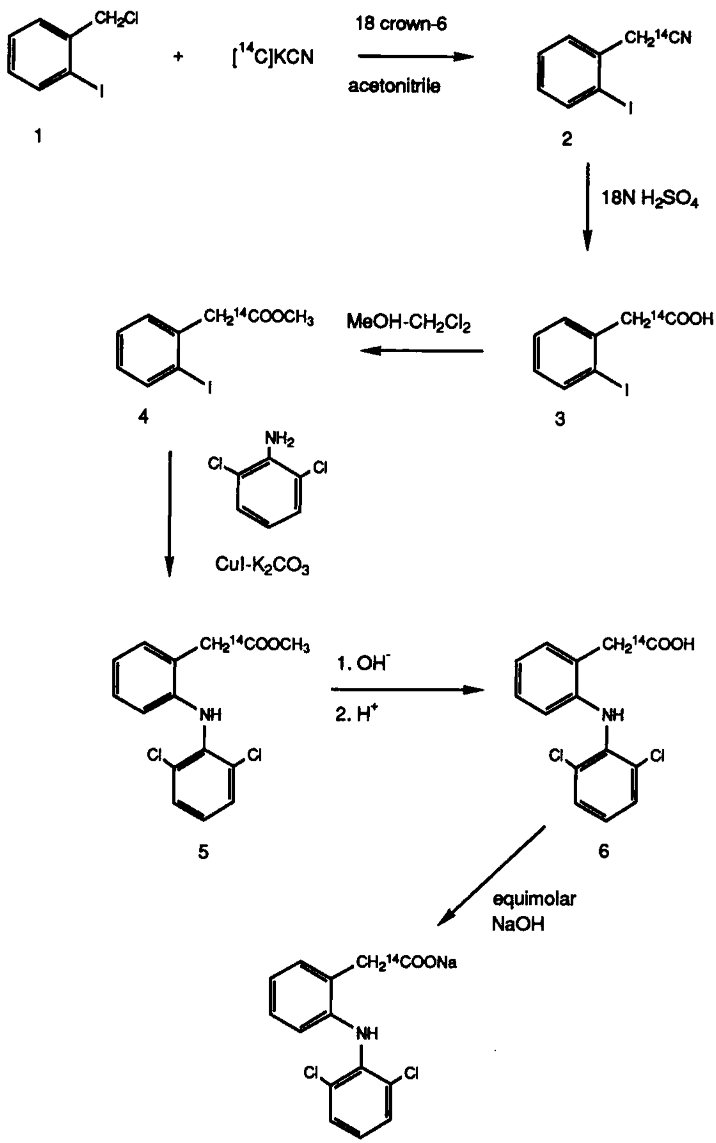


Figure 6. Synthesis of $[^{14}C]$ diclofenac sodium

5 Pharmacology

5.1 Anti-inflammatory Activity

Diclofenac sodium is a potent anti-inflammatory agent, the effects of which have been demonstrated in carrageenan and kaolin paw oedema models of rats with adjuvant arthritis between18 and 21 days following the injection of Freund's adjuvant (18). The $ED_{50}$is many times lower than most anti-inflammatory NSAID's and at least half that of indomethacin and naproxen.

5.2 Inhibition of Prostaglandin Synthesis and Platelet Aggregation

The drug is among the most effective inhibitors of PGE synthetase, acting at a concentration of 1.6 µmol/L (19). It markedly inhibits platelet aggregation in rats (20). Jobin and Gagnon (21) also studied inhibition of ADP and collagen- induced aggregation of human platelets by diclofenac sodium and found it to be a potent and partial inhibitors respectively.

5.3 Analgesic and Anti-pyretic Effects

Following interperitoneal injection of various irritants (p-benzoquinone and aceticacid) to mice and rats respectively, diclofenac was found to have an analgesic effect in the writhing syndrome, an effect much higher than other NSAIDs tested. The anti-pyretic activity was also been shown in with yeast-induced febrile rats (18).

6 Pharmacokinetics, Metabolism and Metabolic Activity

6.1 Absorption,blood plasma concentration and Excretion

Voltaren is completely absorbed from the gastrointestinal tract after oral administration. The half-life of Voltaren is approximately two hours, with mean peak plasma levels of approximately 0.5 µg/ml and 1.0 µg/ml occurring 2-3 hours after single doses of 25 mg and 50 mg of enteric coated tablets, respectively; mean peak plasma levels of 1.9 µg/ml are reached two hours after a single dose of 75 mg (22). Four hours after dosing the levels still detectable in the plasma are equivalent to about 10% of the maximum concentrations. Rectal administration of diclofenac sodium suppositories produces rapid peak plasma concentration at a rate and level of the same order as oral administration of the drug in solution. In rats and dogs, majority of the drug is found in the faeces, indicative of biliary excretion, whereas in rhesus monkeys 76% is excreted via the kidneys. In man renal excretion is greater than biliary excretion (23).

6.2 Synovial Fluid Concentration

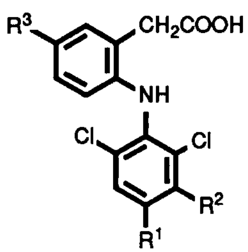
Voltaren penetrates the synovial membrane and diffuses into the synovial fluid. From 4 to 24 hours after dosing, synovial levels of Voltaren are higher than the corresponding plasma levels (24).

6.3 Tissue Distribution

Following rapid absorption, the drug is widely distributed with highest concentrations in the elimination organs (liver and kidney) and in the blood (22).

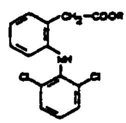
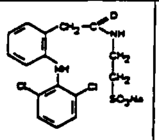
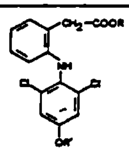
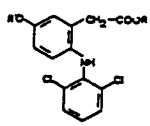
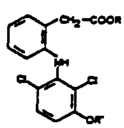
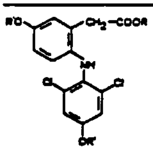
6:4 Metabolic Changes and Activity

In man and monkeys, the metabolic change is hydroxylation , whereas in rats and dogs, the major metabolites are formed by direct conjugation, -Figure 7 - (22).



Metabolite	R_1	R_2	R_3
I	OH	H	H
II	H	H	OH
III	OH	H	OH
IV	H	OH	H

Metabolites I, the 4-hydroxy derivative is the main metabolite in man. The metabolite I and IV reduce paw oedema and inhibit prostaglandin synthetase but all are at least 30 X less effective in the oedema test than diclofenac sodium on a dose/effect basis. The effects of metabolites II and III are produced only by high dosage and are not relevant. Metabolite I is about 6x more active than aspirin in the kaolin oedema test and also effective in rat adjuvant arthritis, though diclofenac itself is 40x more potent. All four have moderate analgesic activity in the writhing test but only metabolite I has an antipyretic effect. In acute toxicity studies, metabolite I has an LD_{50} similar to diclofenac, the others (II, III, IV) having much higher levels (22). A new metabolite, 3'-hydroxy- 4'-methoxy diclofenac has been identified, however, the metabolite does not significantly contribute to the therapeutics effect of the drug (25).

	TYPE OF METABOLITES		URINE	BILE
	CONJUGATES OF DICLOFENAC	RAT DOG MONKEY BABOON MAN	• ● ●● ●● ●	★ ★★★ •
	DICLOFENAC TAURINE CONJUGATE	DOG	●●●	
	CONJUGATES OF THE 4'-HYDROXY DERIVATIVE OF DICLOFENAC IN RAT URINE R' = (SO_3H)	RAT DOG MONKEY BABOON MAN	●●● • ●● ●●● ●●●	●● • ●●
	CONJUGATES OF THE 5-HYDROXY DERIVATIVE OF DICLOFENAC	RAT DOG MONKEY BABOON MAN	● • ●●● ●● ●	● • ●
	CONJUGATES OF THE 3'-HYDROXY DERIVATIVE OF DICLOFENAC	MONKEY BABOON MAN	● ● •	 •
	CONJUGATES OF THE 4',5-DIHYDROXY DERIVATIVE OF DICLOFENAC	RAT MONKEY BABOON MAN	• ● ● ●	• •

• < 5% , ● 5 - 10% ●● 10 - 20% ●●● 20 - 30% ★ 30 - 40% ★★★ 80 - 90% OF THE DOSE

Figure 7. Metabolites of diclofenac sodium and their metabolic pathways.

6:5 Plasma Protein Binding

Using ^{14}C-labelled drug and equilibrium dialysis, diclofenac is reported to be bound to human serum to the extent of 99.7%, of which not less than 99-99.4% is bound to serum albumin fraction. It does not modify the binding of warfarin acenocoumarol, prednisone and salicylic acid to proteins (22).

6:6 Drug Interactions

Concomittant administration of single oral doses of aspirin 600mg/kg and diclofenac sodium (50mg), causes a reduction in the area under the curve (AUC) of the diclofenac plasma profile (26). This was confirmed by Ciba- Geigy Ltd (27). Further studies in rat by Ciba Geigy (28) showed that proportion of unbound diclofenac increased with salicylate dosage and that the total (bound plus unbound) diclofenac in the plasma increased while the excretion of the drug and metabolites increased in the bile. Rosak and Schoffling (29); and Chlud (30) reported that insulin blood sugar and plasma tolbutamide levels were not affected by diclofenac sodium. Studies also showed that diclofenac sodium do not interact significantly with oral anticoagulant like acenocoumarol (31).

6:7 Elderly Patients

Pharmacokinetic studies carried out by Willis and Kendall (32), in young females (18 to 21 years) and elderly females (62 to 78 years) dosed with 50 mg of diclofenac sodium revealed that individuals mean curves and statistical analysis of drug handling in the two groups was similar. The amount of free drug excretion appeared greater in the younger group but the differences were not significant. Ciba Geigy Basle (33) compared the AUCs from a single dose of 50mg and repeated doses of 2 X 50 mg/day for 4 weeks in younger and older subjects and reported that differences between the groups were not statistically significant. In neither group did the bioavailability change over the period studied.

7:0 Toxicity Studies

The major side effects of NSAIDs are gastric irritation and ulceration, due to inhibition of cyclooxygenase. Cyclooxygenase, PGE_2 has a cytoprotective effect on the gastric mucosa by inhibiting gastric acid secretion and by helping to maintain the gastric mucosa barrier (34). In acute toxicity studies diclofenac sodium was found to cause gastric lesions in a lower dose (12 mg/kg) compared to NSAIDs such as phenylbutazone ,

oxyphenbutatozone 620mg/kg. Chronic toxicity studies over a period of 26 weeks (in doses of 5, 15, 75 mg/kl) in rhesus monkeys produced evidence of gastrointestinal lesions only at the highest 75 mg dose (23).

8 Analytical Methods

8.1 Gas-Liquid Chromatography

Gas - liquid chromatography has been used to analyze diclofenac sodium and its metabolites (35-40). The column conditions are presented in Table III. Because of its high sensitivity, electron capture detector is the detector of choice. Before injecting into the column, diclofenac or its metabolites are derivatized into the indolones or the methyl esters.

Table III

Gas - Liquid Chromatography of Diclofenac Sodium

Column	Temperature	Reference
25mx0.3mm id; coated with barium carbonate and statically coated with Carbowax 40M	240°C	39,40
2mx3mm id; 3%OV-17 Gas Chrom Q on 80-120 mesh glass beads	300°C	38
4 ftx3mm id; 3% JXR (methyl silicone) on Gas-Chrom Q	205°C	35
2mx3mm id; 1.5% Silicone OV-17 on Shimalite W AW DMCS, 80-100 mesh.	260°C	36,37

8.2 Thin Layer Chromatography

Thin layer chromatography has been used to analyze diclofenac sodium in suppositories (16) and plasma (41).

8.3 High Performance Liquid Chromatography

Determination of diclofenac sodium in biological fluids by High Performance Liquid Chromatography were described (42-47). The fluids included plasma (42-46), urine (46) and synovial fluid (44). Detection by UV spectrometer was employed with absorbance at 210nm (43), 215nm (44-46), 280nm (42) and 282nm (46). The detection limits are 5-25ng/ml of fluid. Reverse - Phase C_{18} columns prove to be suitable for diclofenac sodium analysis (Table IV). El-Sayed et al employed a rapid sample preparation by precipitating the serum proteins with acetonitrile instead of using phosphoric acid and organic solvent extraction (42). Grandjean et al designed an automated robotic extraction and subsequent analysis of diclofenac which could handle large number of samples with little manpower (43). Analysis from a solid dosage form (tablet) has also been reported (48).

Table IV

High Performance Liquid Chromatography forDiclofenac Sodium

Column	Mobile Phase	References
Spherisorb RP-C8 column (5 µm)	Acetonitrile-water (50:50, v/v) adjusted to pH 3.3 with glacial acetic acid	42
Spherical C18 (5 µm)	Isopropanol-acetonitrile-0.02M acetate buffer pH 7 (NaCl 0.02M; 5:18:77)	43
Supelcosil LC-18 (5 µm)	Methanol-acetonitrile-0.02M sodium acetate buffer (25:20:55)	44,45
Nucleosil C18 (10 µm)	Methanol-acetonitrile-pH 7 phosphate buffer(30:17:53; v/v)	46
µmbondapak C 18 (30 cm x 3.9 cm id)	Methanol 55% in 50mM orthophosphoric acid, pH 4.0	47
Microbondapak CN (30 cm x 3.9 cm id)	Methanol-acetate buffer (65:35% v/v) pH 3.7	48

8.4 Spectrophotometric Method

Diclofenac sodium may be assayed by simple spectrophotometric method at 600nm inwater (49). Diclofenac sodium reacted with 3-methyl-2-benzothiazolinone hydrazone hydrochloride and cerium ammonium sulfate to form a colored complex which exhibited λ_{max} at 600nm. Harland *et al* (50) have also analysed diclofenac sodium spectophotometrically at 275 nm from hydrophilic matrix (polyvinyl alcohol). Other investigations involving analysis by spectrophotometric methods include solubility studies reported by Fini *et al* (51) and ongoing work by Vilivallam and Adeyeye (52). In both cases, the analysis were carried out at 275 nm and 280 nm respectively .

8.5 Nuclear Magnetic Resonance

The proton magnetic resonance (PNMR) method to quantify diclofenac sodium in pure and tablet forms was described (3). The sharp singlet at 3.62ppm which corresponded to the methylene protonsin diclofenac was chosen for quantitative measurement. Anhydrous sodium acetate was used as internal standard. The methyl protons of sodium acetate gave a sharp singlet at 1.81ppm. The amount of diclofenac could be calculated by comparing the peak ratio of diclofenac to that of the internal standard since the amount of internal was known. The PMR spectrum could also be used to examine the purity of the drug.

8.6 Colorimetric analysis

Sane *et al* described a method for determining diclofenac sodium from pharmaceutical preparation by reacting diclofenac sodium with potassium ferricyanide in the presence of sodium hydroxide to form a yellow complex which showed maximum absorbance at 450 nm (53). Agrawal *et al* described a rapid method for the determination of diclofenac sodium. and reported that the drug solution turned yellow color when reacted with sodium nitrite and hydrochloric acid and exhibited maximum absorbance at 390 nm. Beer's law was followed in the range of 50-600 µg/ml (54).

8.7 Gas chromatography - Mass Spectrometry

Gas chromatography - Mass spectrometry is the most sensitive method reported in the analysis of diclofenac sodium (1). The lowest limit of detection of diclofenac is 0.2 ng/ml of plasma which is 10 times more sensitive than using gas chromatography alone.

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ANALYTICAL PROFILE OF DIETHYLSTILBESTROL

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CONTENTS

1. DESCRIPTION

1.1 Nomenclature

1.1.1 Chemical Name

3,4-Di(p-hydroxyphenyl)hex-3-ene.
3,4-Bis(p-hydroxyphenyl)-3-hexene.
4,4(1,2-Diethyl-1,2-ethenediyl)bis(E)phenol.
-4,4′-Dihydroxy-α,β-diethylstilbene.
α,α′-Diethylstilbenediol.
α,α′-Diethyl-(E)-4,4′-Stilbenediol.
trans-α,α′-diethyl-4,4′-Stilbenediol (1-4).

1.1.2 Generic Names

Diethylstilbestrol; Diethylstilboestrol; DES, Stilbestrol; Stilboestrol; Stilbol (2,3,5).

1.1.3 Properietary Names

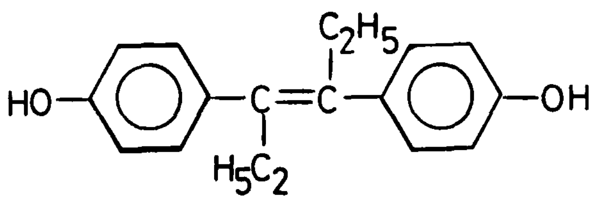
Antigestil; Bio-des; Biofon; Cyren A; Distilbene; Domestrol; Estrobene; Estrosyn; Fornatol; Grafestrol; Hi-Bestrol; Microest; Neo-Oestranol 1, Oestrogenine; Oestromenin; Oestromensyl; Oestromon; Palestrol; Percutacrine Oestrogenique Iscovesco; Serral; Sexocretin; Sibol; Stilbetin; Stilboefra; Stilboestroform; Stilkap; Synestrin (Tablets); Synthoestrin (1-5).

1.2 Formulae

1.21 Empirical

$C_{18}H_{20}O_2$

1.22 Structural



1.23 CAS registry No.

[56-53-1].

1.3 Molecular Weight

268.4

1.4 Elemental Composition

C 80.56%, H 7.51%, O 11.92%.

1.5 Appearance, Color and Odor

White or almost white, crystalline powder and is odorless (2,4).

2. PHYSICAL PROPERTIES

2.1 Solubility

Very slightly soluble in water, soluble in aqueous solutions of alkali hydroxides, soluble in 3 parts of ether, in 5 parts of 95% v/v alcohol and in 40 parts of arachis oil, slightly soluble in chloroform (2,4).

2.2 Melting range

169-175^{o}C (1).

2.3 Spectral Properties

2.3.1 Ultraviolet Spectrum

The ultraviolet spectrum of diethylstilbestrol in 0.1N sodium hydroxide, maximum at 259 nm (E1%, 1 cm 764). (2). The ultraviolet spectrum of diethylselbestrol in ethanol is obtained using Cary, 219 spectrophotometer and is shown in Figure [1]. The spectrum shows a major band at 240 nm and a minor band at 280 nm.

2.3.2 Infrared Spectrum

The infrared spectrum of diethylstelbestrol as KBr disc is presented in Figure [2] and is recorded in Perkin-Elmer spectrophotometer model 580 B. The structural

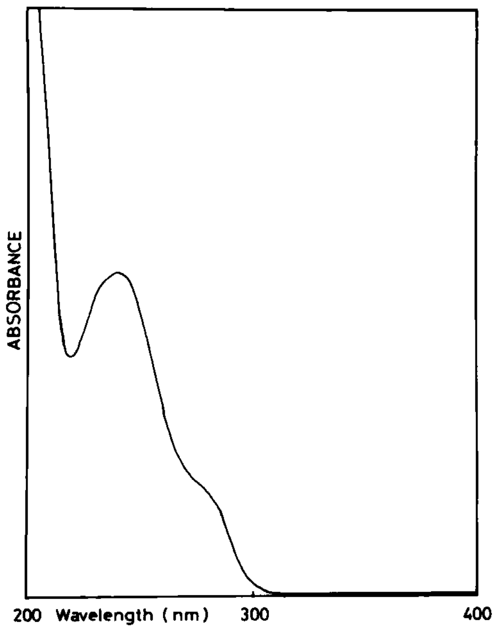


Figure 1: Ultraviolet spectrum of Diethylstilbestrol in ethanol.

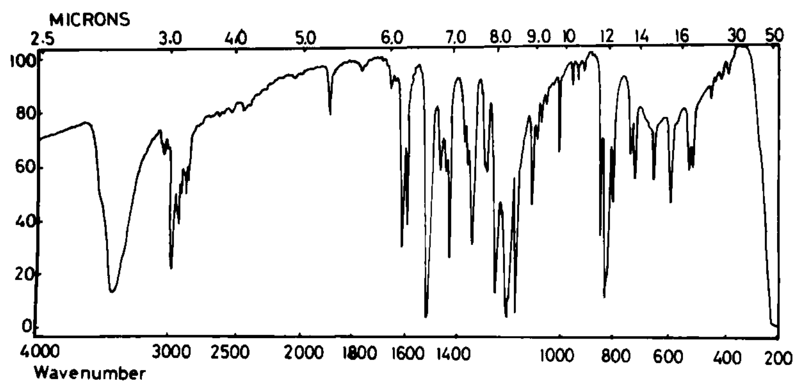


Figure 2: Infrared spectrum of Diethylstilbestrol, KBr disc.

assignments have been correlated with band frequencies and are given in the following table :-

Frequency (cm^{-1})	Assignment
1592,1612	C=C stretch (aromatic)
2990	C-H stretch
3420	OH stretch
1340	C-H bending
830	C-H bending (aromatic)

Clarke (2) reported the following principal peaks as KBr disc: 1198, 1515 and 1165 cm^{-1}.

2.3.3 Nuclear Magnetic Resonance Spectra

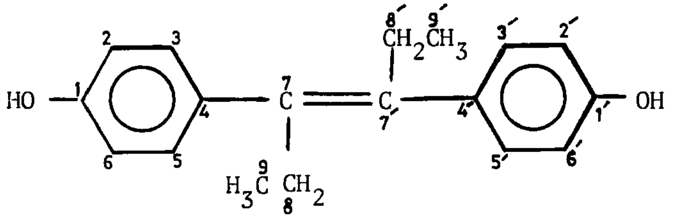
2.3.3.1 Proton Magnetic Resonance Spectrum (PMR)

The PMR spectrum of diethylstilbestrol was recorded on varian T-60 A spectrometer with DMSO-d_6 as a solvent and TMS (tetramethylsilane) as internal reference. The spectrum is shown in Figure [3] and the signal are assigned as follows:-

Proton	Chemical shift (ppm)	Multiplicity
Phenyl protons	6.6 - 7.1	Multiplet
CH_3	0.65	Triplet
CH_2	2.05	Quartet
OH	9.16	

2.3.3.2 Carbon-13 NMR Spectra

The Carbon-13 NMR noise-decoupled and off-resonance spectra are presented in Figures [4] and [5] respectively. The samples were dissolved in DMSO-d_6 and the spectra were obtained on Jeol-XL-100 NMR spectrometer using tetramethylailane (TMS) as internal reference standard. Spectral assignments are listed below:



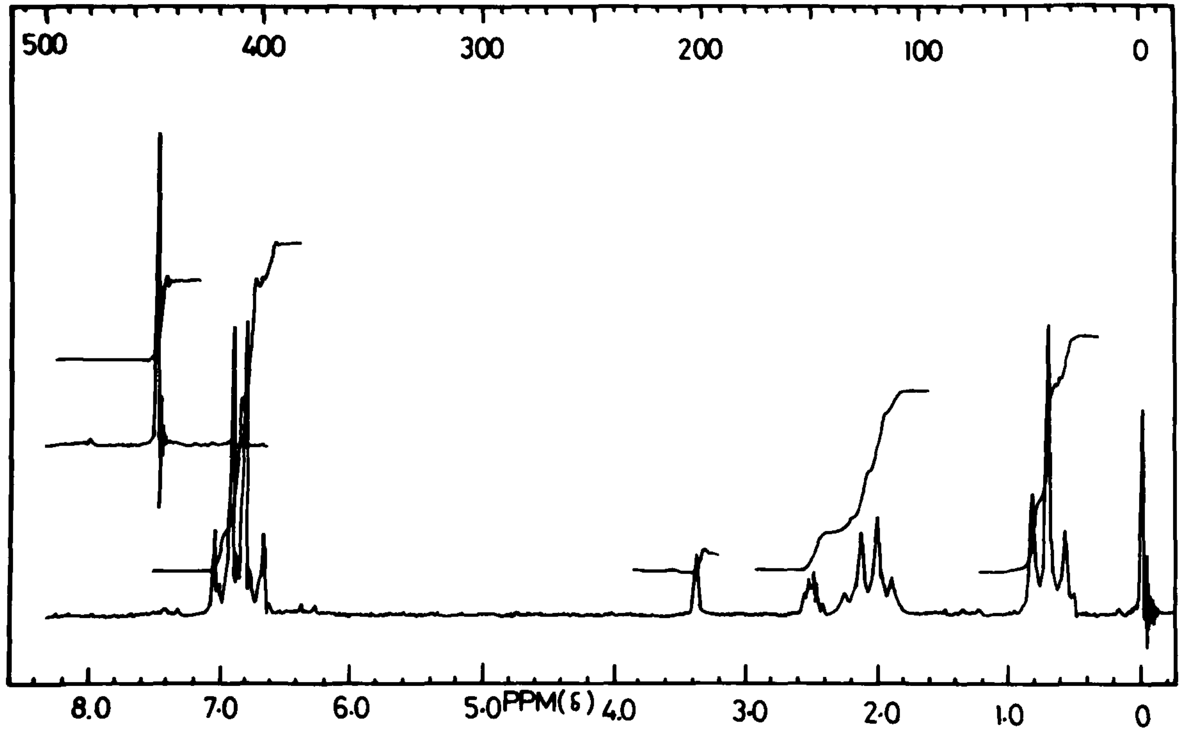


Figure 3: Proton nuclear magnetic resonance spectrum of Diethylstilbestrol in DMSO-d_6 using TMS as reference standard.

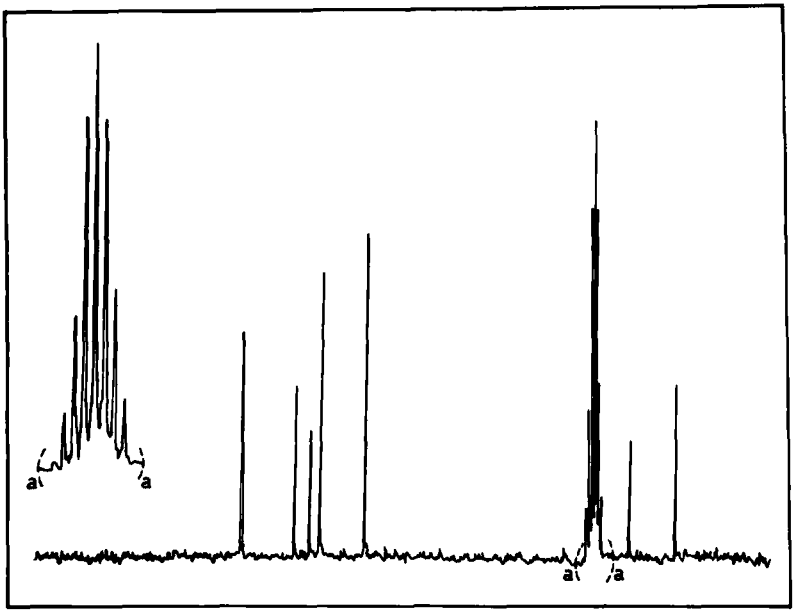


Figure 4: Proton-decoupled carbon-13 NMR spectrum of Diethylstelbestrol in DMSO-d_6 using TMS as reference standard.

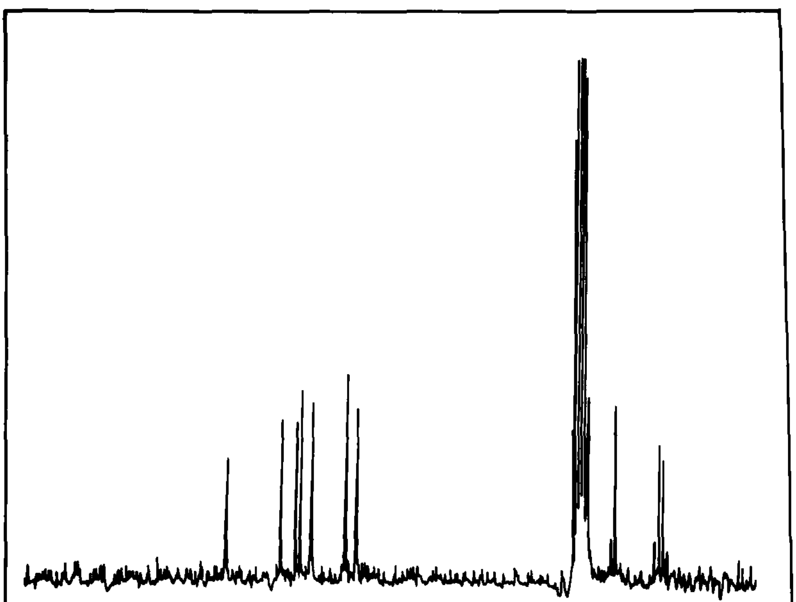


Figure 5: Off-Resonance carbon-13 NMR spectrum of Diethylstelbestrol in DMSO-d_6 using TMS as reference standard.

Carbon No.	Chemical Shift (ppm) relative to TMS	Multiplicity
1 and 1´	155.58	Singlet
2,2´ and 6,6´	114.78	doublet
3,3´ and 5,5´	129.22	doublet
4 and 4´	137.85	singlet
7 and 7´	132.45	singlet
8 and 8´	28.00	triplet
9 and 9´	13.27	quartet

2.3.4 Mass Spectrum

The electron impact (EI) mass spectrum at 70 eV recorded on Varian Mat 311 mass spectrometer and the methane-derived chemical ionization (CI) mass spectrum obtained with Finnigan 4000 mass spectrometer are shown in Figures [6] and [7] respectively. Scheme (1) shows the proposed fragmentation pathway.

Low resolution mass spectra for diethylstilbestrol, dienestrol, hexestrol, the acetates of them, dimethyl, the bis-(trimethylsilyl) ethers, and some deuterated derivatives of these were studied by Engel et al (6).

2.4 X-Ray Chrystallography

Simley and Rossmann (7) have determined the crystal structure of diethylstilbestrol because of the interest in its biological activity. Crystal of the drug are orthorhombic with a = 19.18, b = 5.32 c = 15.01 A., Z = 4, calculated d = 1.164, experimental d = 1.162, and space group Pcab. The planes of the two benzene rings were paralleled but not coplanar. This loss of coplanarity resulted from steric effect between the methylene carbon of the ethyl group and the ortho-hydrogen atoms.

The bond length A and angles (o) are listed below (Table 1) and Figure [8] gives a respective view of the molecule.

Busetta and Hospital (8) have studied the crystalline structure of diethylstilbestrol. The drug was crystallized by sublimation in the form of orthorhombic needles, space group Pbca with a = 18.992 ± 0.005, b = 14.931 ± 0.005 and c = 5.296 ± 0.005 A; Z = 4. The unit

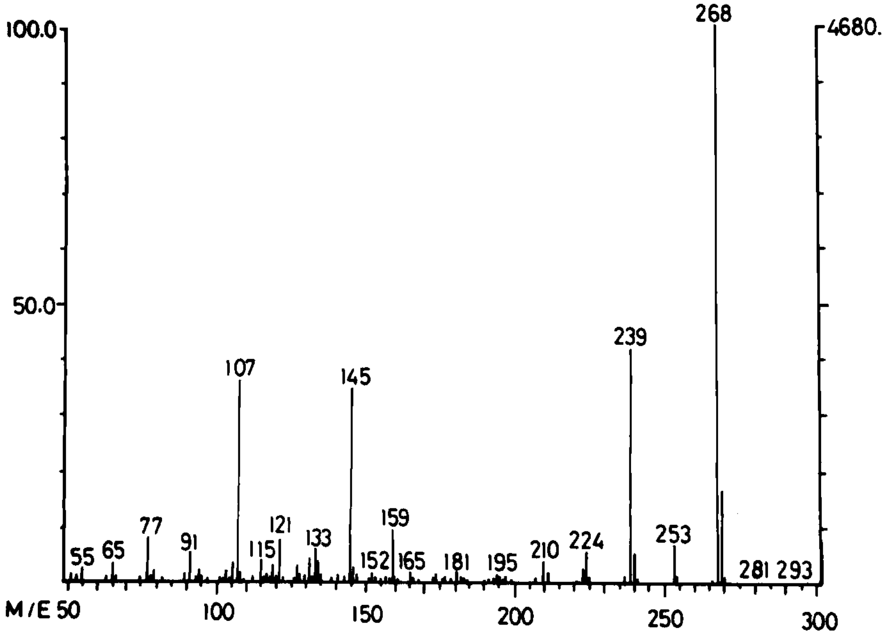


Figure 6: Electron impact (EI) mass spectrum of Diethylstilbestrol.

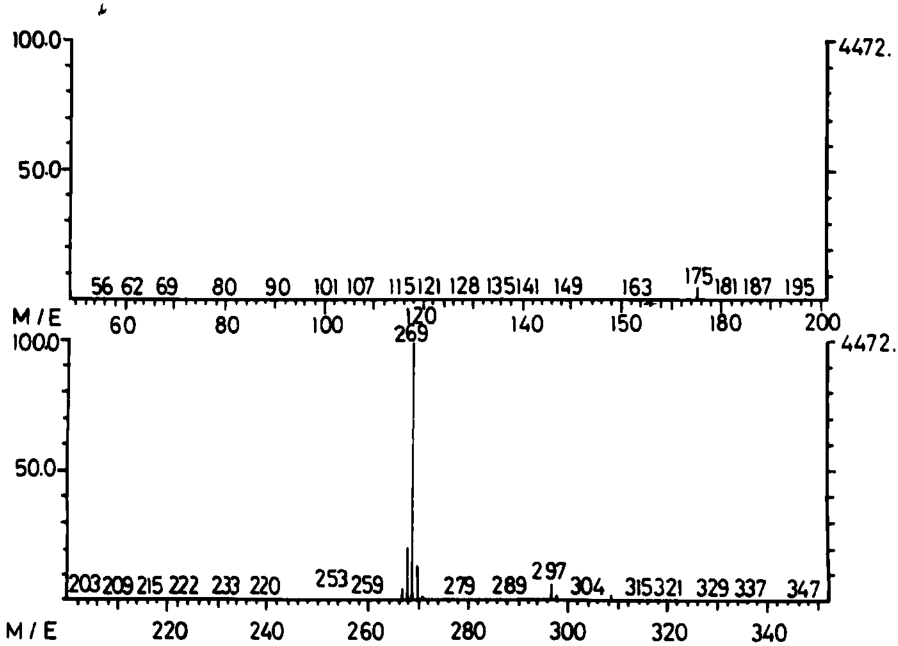
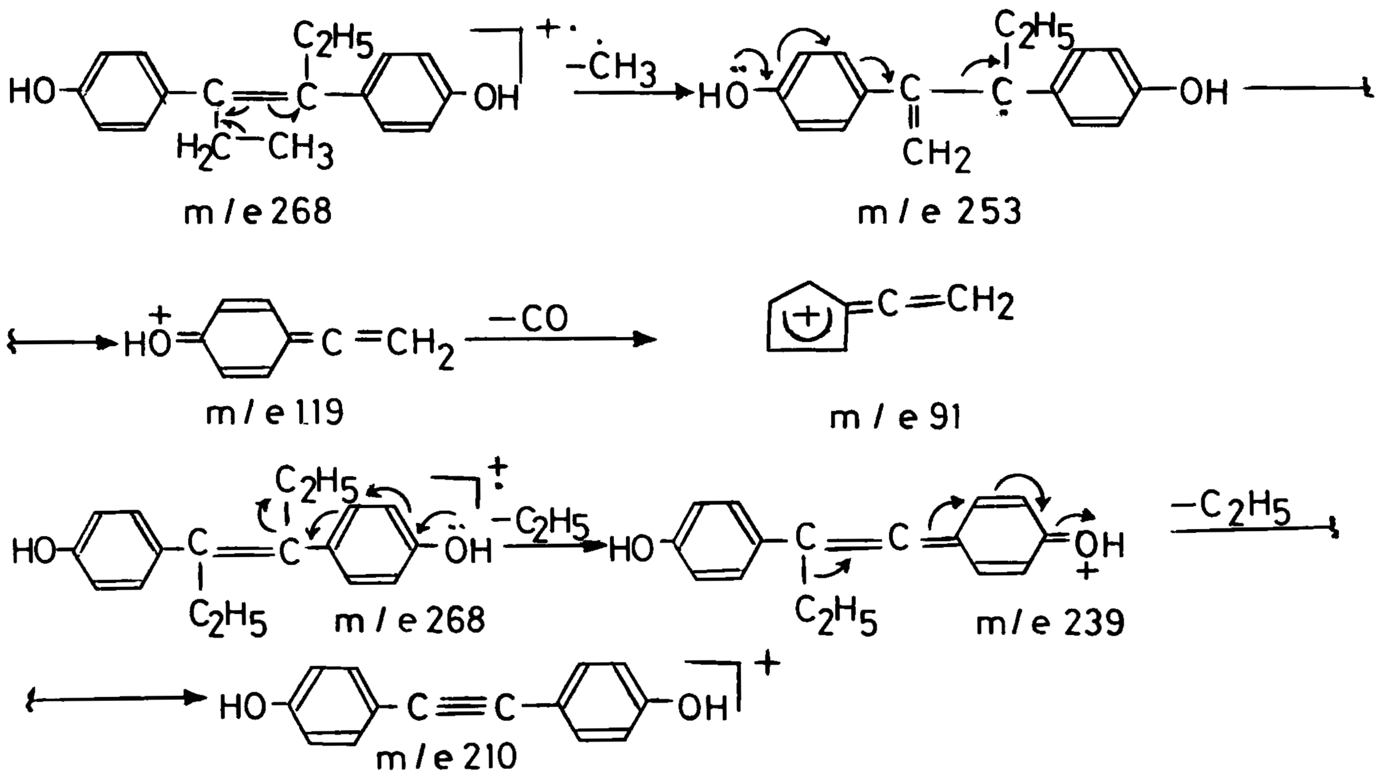
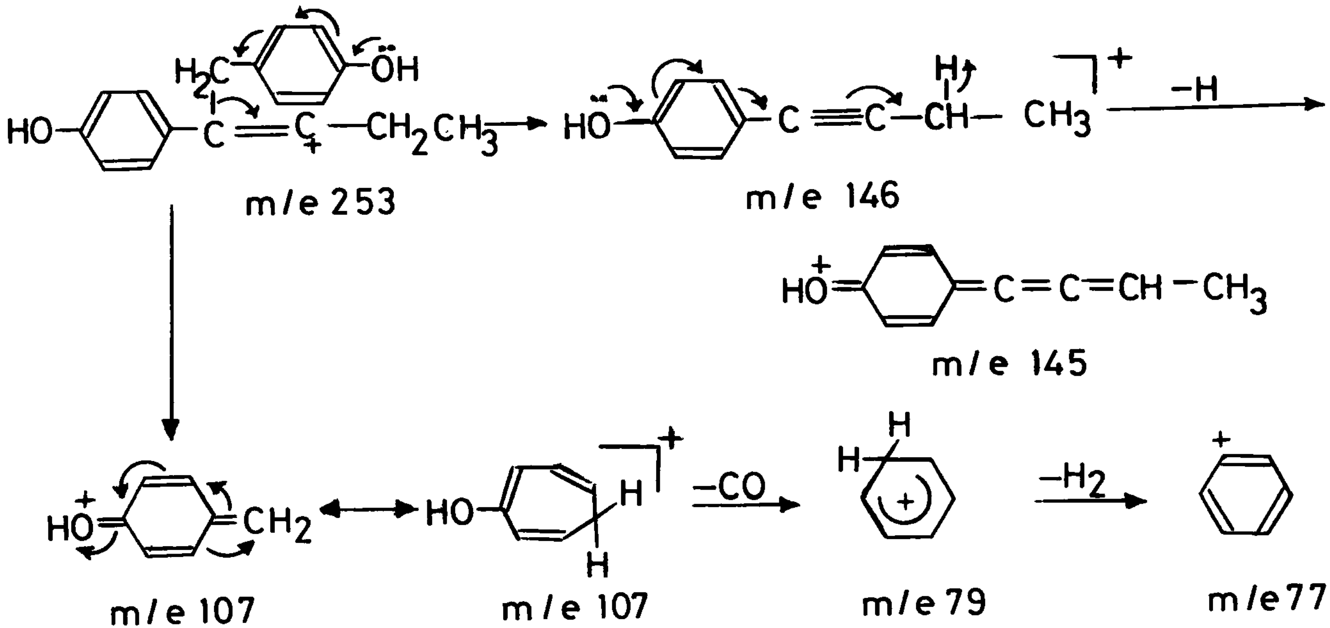


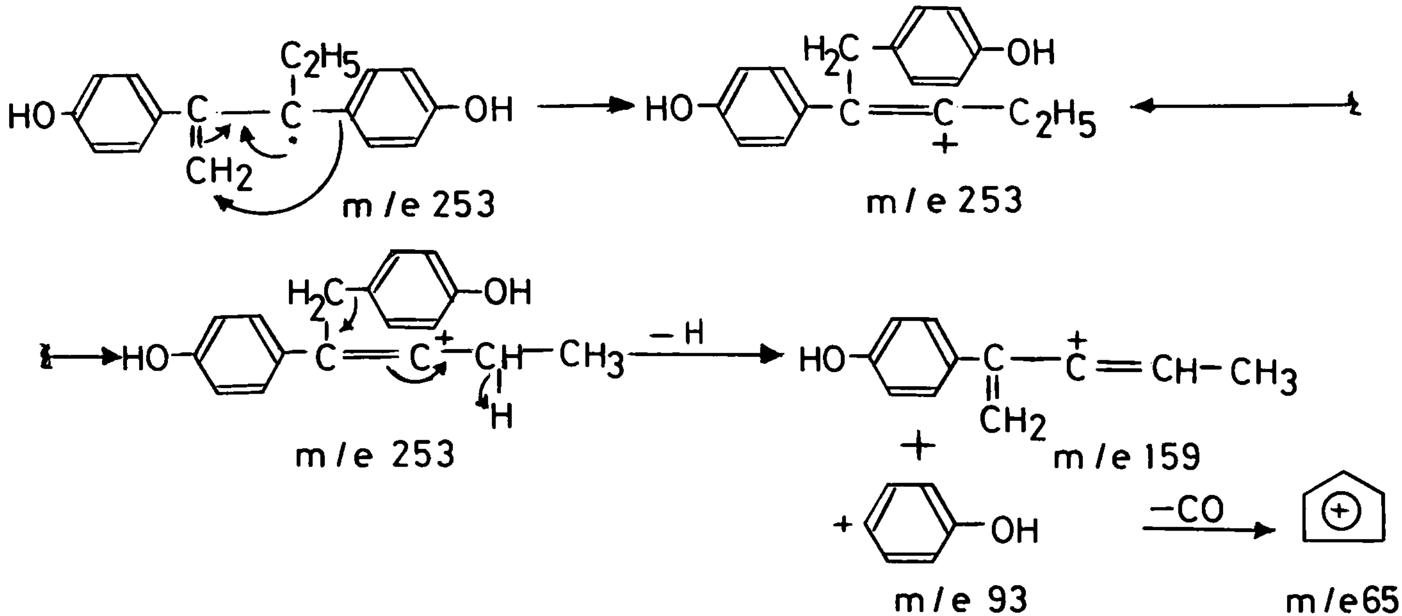
Figure 7: Chemical ionization (CI) mass spectrum of Diethylstibestrol.



Scheme 1: Proposed fragmentation pathway of diethylstilbestrol.



Scheme 1: Proposed fragmentation pathway of diethylstilbestrol (continued).



Scheme I: Proposed fragmentation pathway of diethylstilbestrol (continued).

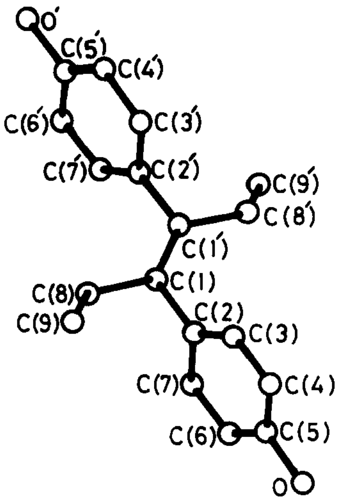


Figure 8: Molecular stereochemistry of $\alpha\alpha'$- diethylstilbene-4,4'-diol.(7)

of symmetry is half a molecule, and the molecules are bonded to each other with hydrogen bonds 3A long and with a dihedral angle of 88°.

Table (1) Bondlength A and Bond angles (o)

C(1)-C(1´)	1.33	C(1´)-C(1)-C(8)	124.07
C(1)-C(2)	1.50	C(1)-C(8)-C(9)	109.32
C(2)-C(3)	1.40	C(1)-C(1)-C(2)	122.22
C(3)-C(4)	1.40	C(2)-C(1)-C(8)	113.78
C(4)-C(5)	1.42	C(3)-C(2)-C(7)	116.05
C(5)-C(6)	1.36	C(2)-C(3)-C(4)	125.15
C(6)-C(7)	1.41	C(3)-C(4)-C(5)	116.27
C(2)-C(7)	1.42	C(4)-C(5)-C(6)	122.65
C(1)-C(8)	1.54	C(5)-C(6)-C(7)	119.15
C(8)-C(9)	1.56	C(2)-C(7)-C(6)	121.65
C(5)-O	1.44	C(4)-C(5)-O	119.57
		C(6)-C(5)-O	117.80

The crystal structure of diethylstilbestrol was also studied in respect to estrogenic activity by Weeks *et al* (9). The drug (single crystals) were grown by slowly cooling a solution of the drug in a 0.01 M solution of p-chlorophenol in iso-octane. The molar ratio of p-chlorophenol of the drug in this solution was 2:1. The systematic absences (OKl for K odd, hOl for l odd, and hKO for h odd) in the diffraction pattern, were consistant with the orthorhombic space group Pbca and the crystal data are :

a = 18.954 $\pm$ 0.004, b = 14.929 $\pm$ 0.001
c = 5.291 $\pm$ 0.001 A (at 20°C, Cu Ka_1 = 1.5045 A)
V = 1497.29 A^3, D_m = 1.14 g.cm^{-3}
(by flotation), Z = 4, D_c = 1.19 g.cm^{-3}, u = 6.1 cm^{-1}
Space group Pbca, D^{15} no.61.

The authors have presented a table showing the agreement between the observed and calculated structure factor amplitudes. Table 2 shows a list of the refined atomic coordinates and the thermal parameters.

The interatomic distances and valency angles involving nonhydrogen atoms are shown in Figure [9] (Standard deviation are in the range of 0.006-0.01 A and 0.2-0.7 respectively). An unusually short apparent distance of 1.498 A between C(8) and C(9) result from the large thermal motion of C(9). C-H distance lies in the range

Table 2(a) : Atomic coordinates and anisotropic thermal parameters for the nonhydrogen atoms
Thermal parameters are of the form

$$\exp [2 \ ^{2} (U \ h^{2}a^{*} + 2U_{12}hka^{*}b^{*} + ...)]$$

The standard deviations of the last two figures are given in parentheses.

	/A	Y/B	L/C	U11	U22	U33	U12	U13	U23
C(1)	0.15324(27)	0.03507(33)	-0.0921 (9)	0.0680(30)	0.0474(24)	0.0533(26)	-0.0020(22)	-0.0058(22)	-0.0081(20)
C(2)	0.21313(25)	0.08731(33)	-0.0548 (9)	0.0576(26)	0.0505(24)	0.0548(26)	-0.0056(21)	-0.0069(21)	-0.0056(20)
C(3)	0.21507(22)	0.14632(28)	-0.1462 (8)	0.0445(22)	0.0378(21)	0.0587(26)	-0.0001(17)	-0.0087(19)	-0.0033(18)
C(4)	0.15396(25)	0.15144(31)	-0.3118 (9)	0.0563(27)	0.0523(27)	0.0556(27)	-0.0082(22)	-0.0093(21)	-0.0106(20)
C(5)	0.09949(23)	0.09820(31)	-0.2722 (9)	0.0479(24)	0.0518(25)	0.0581(26)	-0.0071(22)	-0.0011(21)	-0.0102(21)
C(6)	0.09571(23)	0.04043(28)	-0.0669 (8)	0.0555(24)	0.0357(20)	0.0471(23)	-0.0021(18)	-0.0110(19)	-0.0021(17)
C(7)	0.03131(24)	0.01726(25)	-0.0233 (8)	0.0601(23)	0.0291(21)	0.0536(24)	-0.0040(17)	-0.0113(21)	-0.0004(17)
C(8)	0.04616(27)	0.11730(29)	-0.0418 (10)	0.0633(28)	0.0302(21)	0.0811(34)	-0.0042(20)	-0.0140(25)	-0.0012(21)
C(9)	0.07880(32)	0.14524(35)	-0.2873 (12)	0.0818(36)	0.0482(28)	0.0876(41)	-0.0092(26)	-0.0088(30)	-0.0213(27)
C(3)	0.27396(16)	0.20083(20)	-0.1702 (6)	0.0508(17)	0.0456(16)	0.0700(23)	-0.0126(14)	-0.0014(16)	-0.0056(16)

Table 2(b) : Atomic coordinates of the hydrogen atoms.

H(1)	0.1543	-0.0025	-0.2554
H(2)	0.2524	-0.0827	-0.1638
H(4)	0.1707	-0.1876	-0.4613
H(5)	0.0618	-0.1044	-0.3899
H(8A)	0.0849	-0.1348	-0.0820
H(8B)	-0.0118	-0.1470	-0.0545
H(9A)	0.0630	-0.1087	-0.4219
H(9B)	0.1342	-0.1313	-0.2757
H(9C)	0.0804	-0.2026	-0.3422
H9(3)	0.2729	-0.2250	-0.3303

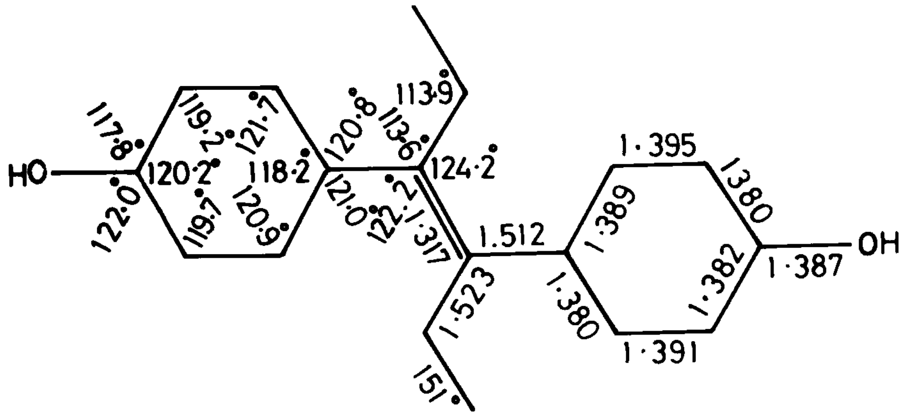


Figure 9: Interatomic distances and valency angles. Corrected for thermal motion (9).

of 0.91 to 1.19 A, with average value of 1.00 A. The O -H distance is 0.92 A.

A close intermolecular contact of 3.03 A occurs between oxygen atoms. The angle OH---O is 171^{o}, and the H----O distance is 2.11 A. The system of hydrogen bonds and the packing of the molecules are illustrated in figure [10] which is a projection of one unit cell down the c axis.

Neither the benzene rings nor the alkyl groups lie in the plane of the central ethylenic linkage. Since the atoms comprising the benzene ring lie nearly in a plane as do those attached to the central double bond, the geometry of the molecule is fixed when the tortional angles about the C(6)-C(7) and C(7)-C(8) bonds are defined. These angles are listed in Table (3). The angle formed by the least-square plane through the ethylenic linkage and the plane through the benzene ring is 62.8^{o}. Since the molecule lie on crystallographic centre of symmetry, the angles of rotation of the two rings are identical. The three dimensional configuration of the molecule is illustrated in Figure [11]. Rotation of the rings out of the central plane give the molecule a thickness of about 4.5 A, which is comparable to the thickness of a steroidal estrogen at C(18). The results of this X-ray investigation confirmed that the synthetic estrogen, diethylstilbestrol has a non planar conformation.

As shown by the distance between the phenolic oxygen atoms, it is a molecule slightly longer than the neutral estrogens, but the rotation of the benzene rings out of the plane of the central double bond results in a molecular dimention similar to the thickness of a steroidal estrogen.

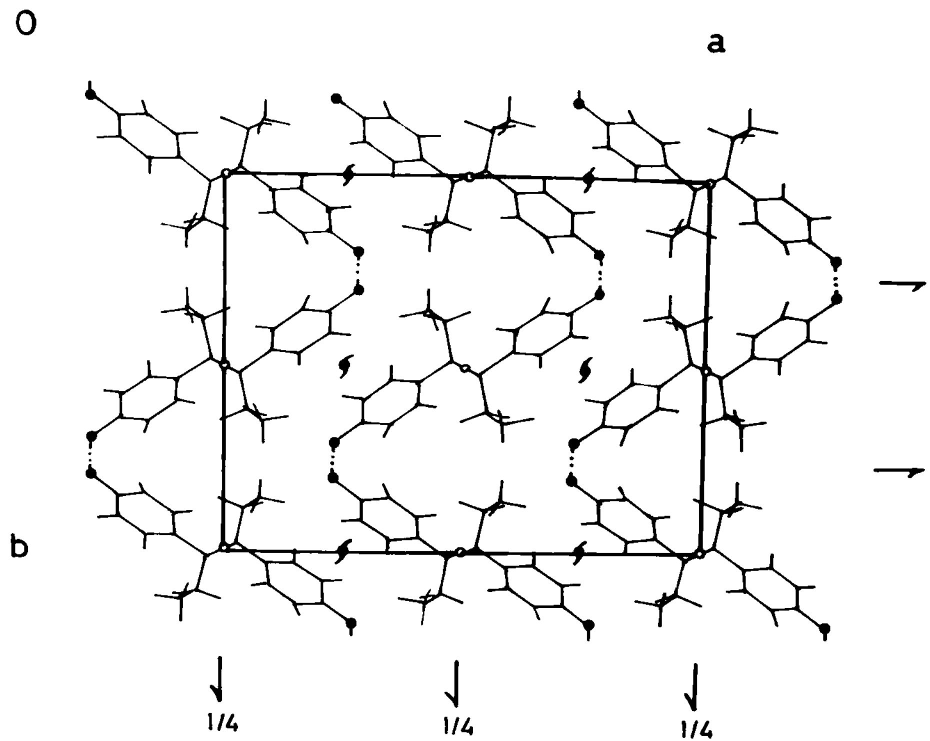


Figure10:Molecular packing seen in projection onto (001). Hydrogen bonds are indicated by dotted lines. ●=Oxygen (9).

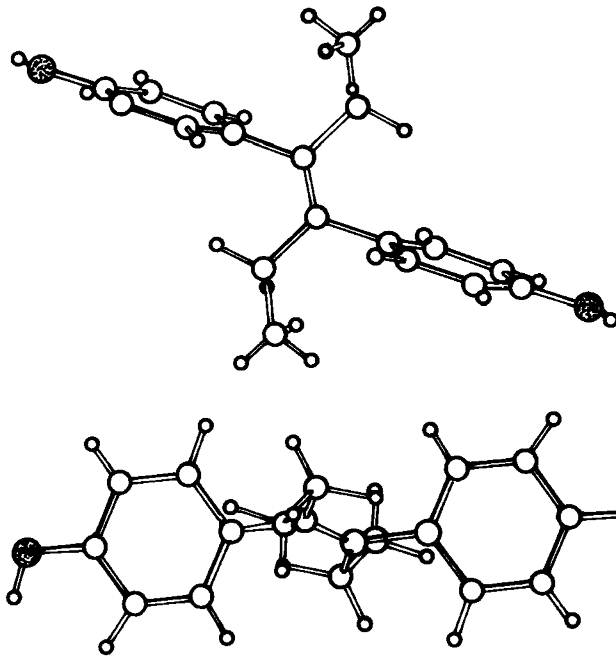


Figure 11: Three dimensional conformation of diethylstilbestrol (9).

Table 3 : Torsional angles for one asymmetric unit of the structure.

	Angle
C(1)-C(6)-C(7)-C(7′)	-118.6^{o}
C(1)-C(6)-C(7)-C(8)	62.1
C(5)-C(6)-C(7)-C(7′)	63.4
C(5)-C(6)-C(7)-C(8)	-115.9
C(6)-C(7)-C(8)-C(9)	56.0
C(9)-C(8)-C(7)-C(7‵)	-123.3

The center of symmetry creates angles in the other half of the molecule which have the opposite sign.

2.5 Thermal Analysis

The thermal analysis of diethylstilbestrol was done between $100^{o}C$ and $250^{o}C$ at a heating rate of 10^{o}/minute (Figure [12]). Purity of sample was found to be 99.86%. Heat of fusion of the sample was found to be 31.9 Km/mole (7.62 Kcal/mole).

2.6 X-Ray Powder Diffraction

The X-ray diffraction patterns of diethylstilbestrol was determined using Philips full automated X-Ray diffraction Spectrogoniometer equipped with PW 1730/10 generator. Radiation was provided by a copper target (Cu anode 2000 w, γ = 1.5480 A) high intensity x-ray tube operated at 40 Kv and 35 mA. The monochromator was a curved single crystal one (PW 1752/00). Divergance slit and the receiving slit were 1 and 0.1^{o} respectively. The scanning speed of the goniometer (PW 1050/81) used was 0.02-2θ per second. The instrument is combined with Philips PM 8210 printing recorder with both analogue recorder and digital printer. The goniometer was aligned using silicon sample before use. The x-ray pattern of Diethylstilbestrol is presented in Figure [13]. The interplanner distances d(A) and relative intensity I/Io are shown in the following table:-

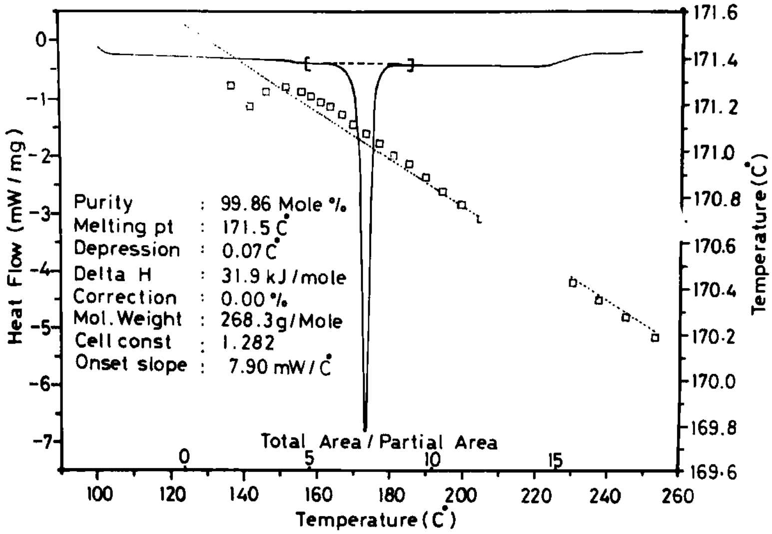


Figure 12: Thermal analysis of diethylstilbestrol

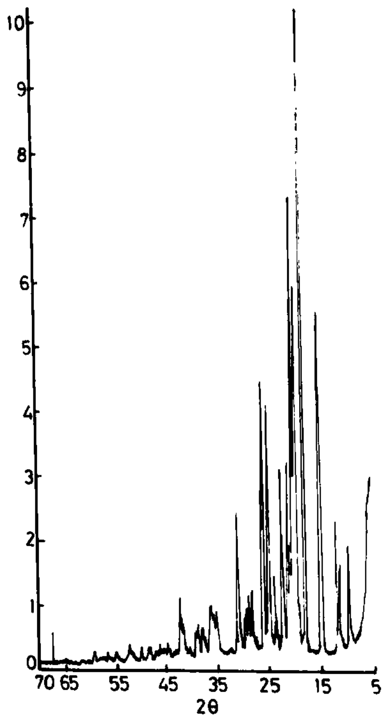


Figure 13: The X-Ray diffraction pattern of diethylstilbestrol

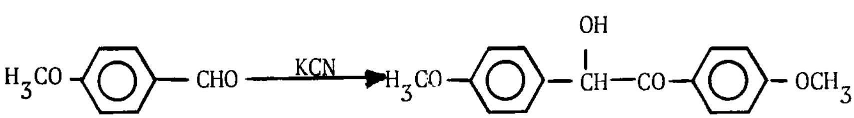
X-Ray Diffraction Patterns of Diethylstilbestrol

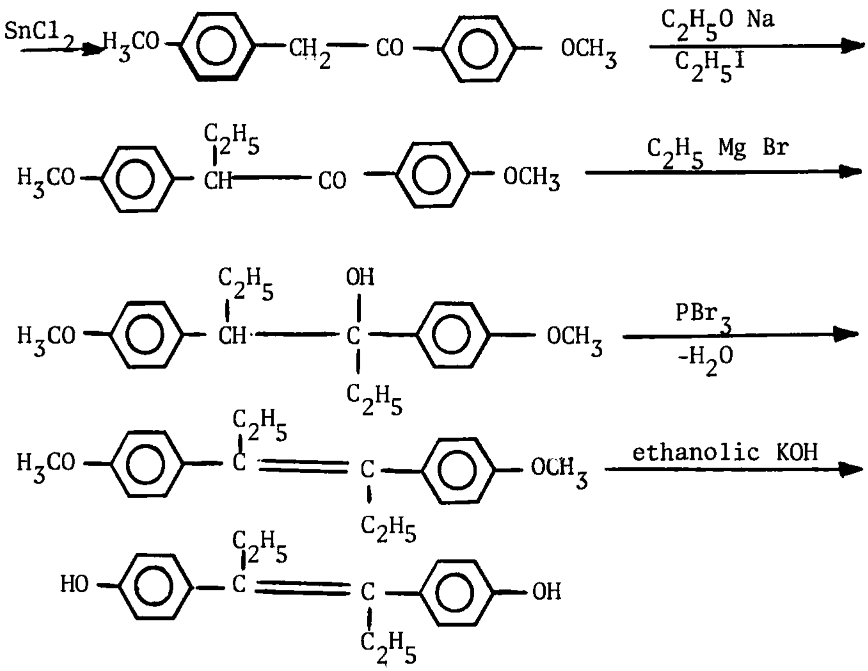
d(A)	I/Io	d(A)	I/Io
9.54	16.40	2.50	9.47
8.06	14.03	2.41	4.42
7.51	20.14	2.38	6.59
5.90	48.03	2.32	6.22
4.85	100.00	2.30	5.44
4.54	51.49	2.24	3.28
4.43	65.06	2.20	3.84
4.33	18.11	2.17	6.29
4.22	27.88	2.14	9.91
4.01	17.98	2.10	3.08
3.94	27.23	2.03	3.54
3.74	12.93	2.00	3.04
3.57	35.93	1.96	3.14
3.44	39.11	1.93	2.93
3.39	22.00	1.89	3.14
3.17	11.35	1.88	3.38
3.10	10.49	1.83	3.49
3.05	7.97	1.75	3.80
3.03	8.02	1.68	2.48
2.91	20.58	1.63	2.42
2.85	5.27	1.56	2.66
2.75	3.35	1.39	1.40
2.56	8.31	1.38	1.37
2.52	7.46		

3. SYNTHESIS

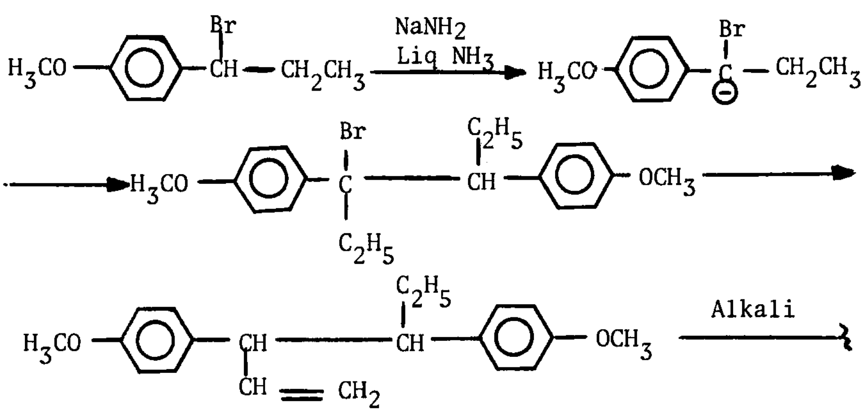
Several methods have been reported for the synthesis of diethylstelbestrol:-

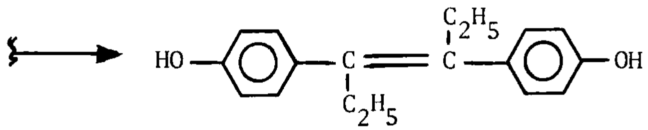
1) The drug was first prepared by Dodds et al (10 a,b) according to the following scheme:-



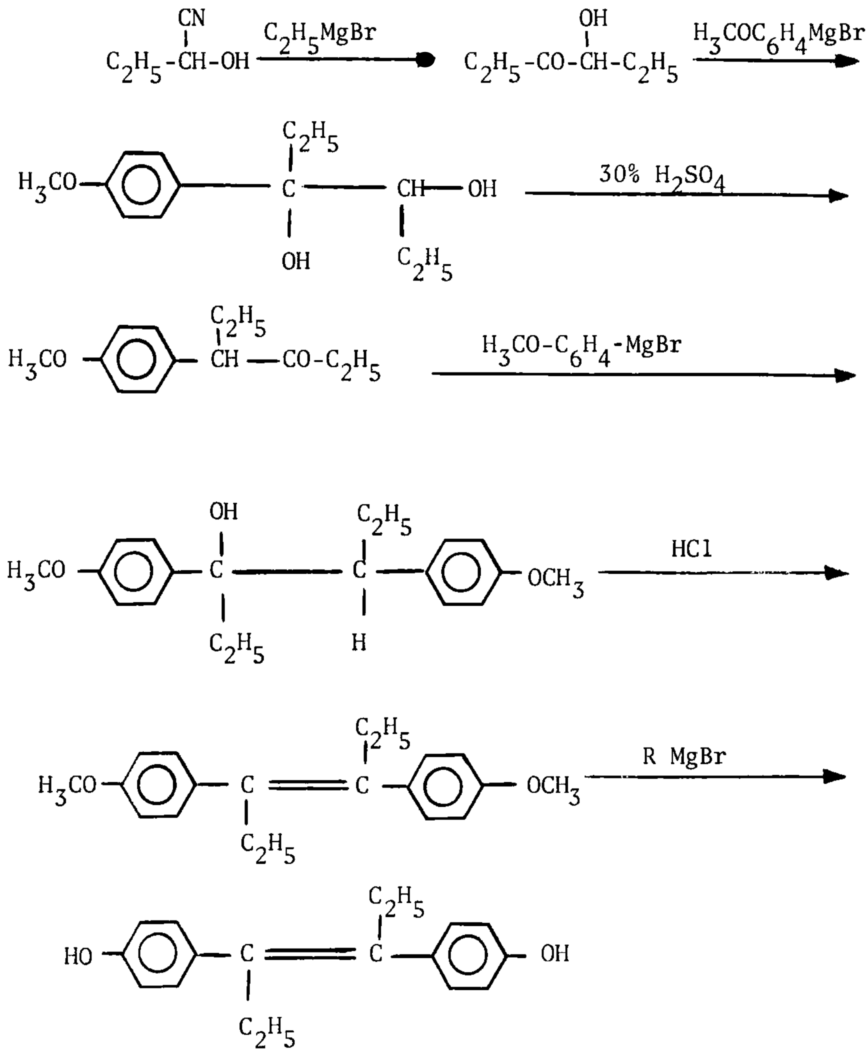


2) Kharasch and Kleiman (11) prepared the drug by treating anethole hypobromide with sodamide in liquid ammonia to form a carbanion which react with unreacting starting material and then the elimination of hydrogen bromide forms an intermediate which on demethylation give diethylstilbestrol.

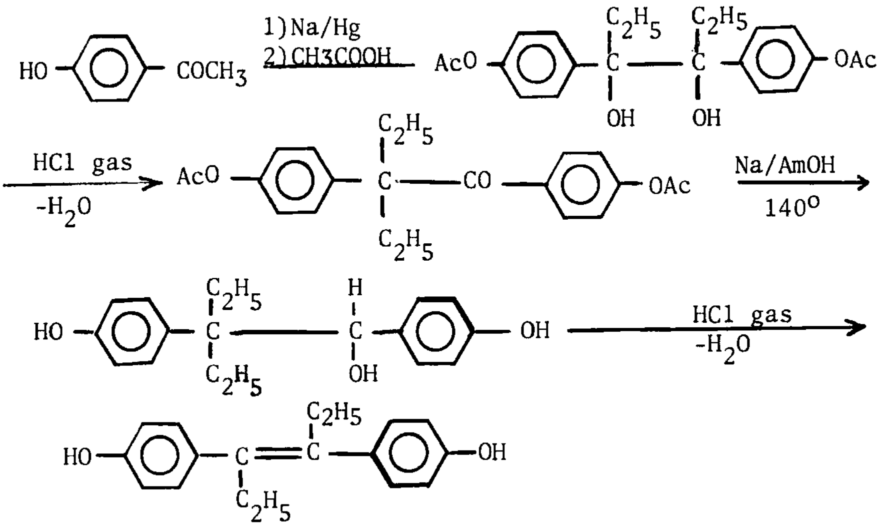




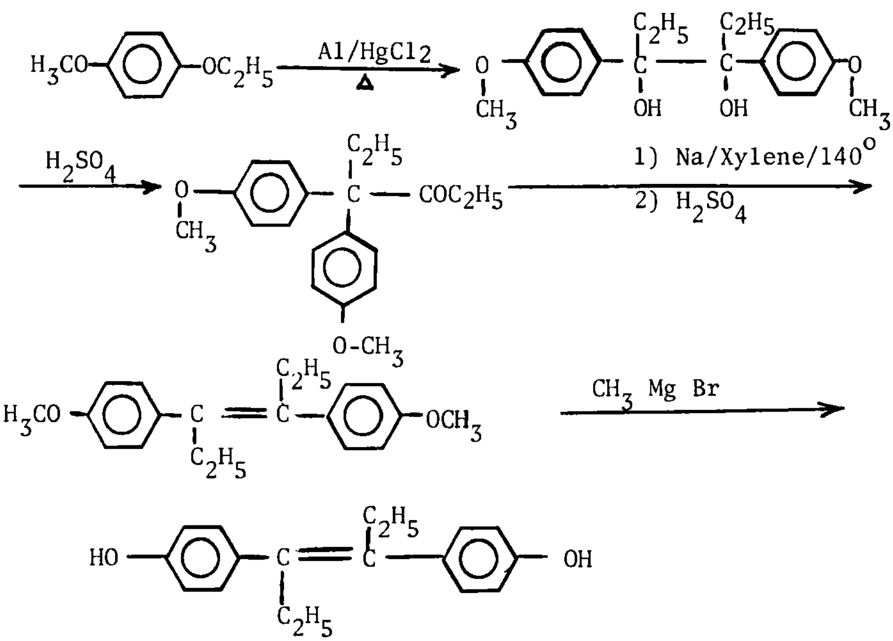
3) Kuwada *et al* (12) have prepared the drug according to the following scheme :-



4) Alder et al (13) have reported the synthesis of diethylstilbestrol according to the following scheme:-

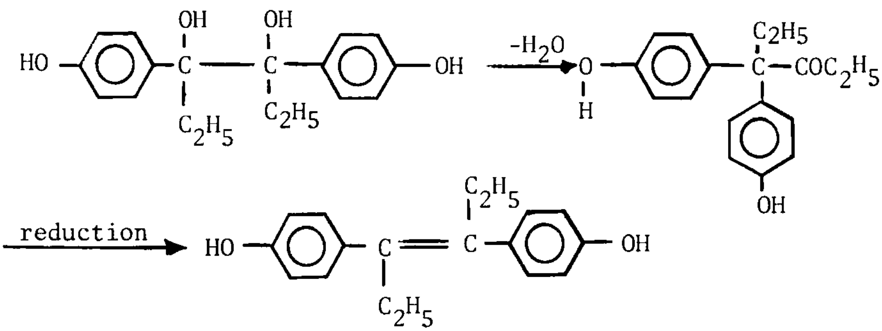


5) Shishido and Nozaki (14) reported the synthesis of diethylstilbestrol through the pinacol-pinacolone compounds according to the following scheme:



6) Yoshida and Akagi (15) have reported the synthesis of the drug by treating p-$CH_3OC_6H_4CHEtC(OH)$-$EtC_6H_4OCH_3$-P with $POCl_3$ in boiling toluene.

7) A method for the synthesis of diethylstilbestrol have been reported by Slager (16). It involves the conversion of 3,4-bis-(p-hydroxyphenyl)-3,4-hexandiol to 3,3-bis(p-hydroxyphenyl)-4-hexanone, treatment with Et_4NBr and Et_4NOH and electrical reduction to give the drug.



4. PHYSIOLOGY, PHARMACOLOGY AND USES

Diethylstilbestrol is a synthetic nonsteroidal estrogenic compound that exhibits the characteristic physiological actions of the hormonal class estrogens, these hormones are largely responsible for the changes that take place at puberty in girls and they go a long way for the tangible and intangible attributes of femininity (17). They affect the body, building and function; the biological role, the physiology, the thinking and psychology of the female. Diethylstilbesterol is indicated clinically for purposes where estrogenic effects are needed. On molecular basis, 1.6 mg. of diethylstilbestrol diphosphate is approximately equivalent to 1 mg. of diethylstilbestrol. The drug is indicated for the following clinical purposes:

1) Cancer treatment

Diethylstilbestrol exerts the usual inhibitory effects of estrogens on androgen dependent carcinomas and also exerts a direct cytotoxic action due to release of free stilbestrol within the neoplasm (18,19,20). Such cancers include prostatic cancer where the level of the

hormone is prostatic tissues was 100 times greater than that in the fat and muscle (20,21). The hormone also suppresses pituitary Lutenizing hormones and hence suppression of androgenic stimuli (20-23).

2) Suppression of Lactation

Diethylstilbestrol is used to suppresses lactation particularly post-partum lactation and galactorrhea (24-26).

3) Polycythemia Vera Treatment

Wirth (27) used diethylstilbesterol to improve cases of polycythemia vera.

4) Symptomatic Relief of Menopause

Diethylstilbesterol has been used with success in symptomatic relief of menopause. It is reported that diethylstilbesterol supplementation may delay the onset of oesteo porisis in post-menopausal women (28,29).

5) Post-hysterectomy Use

Diethylstilbesterol like other estrogens is a definite indication for post hysterectomy menopause to prevent vaginal atrophy and hot flashes (29-31).

6) Contraception

Diethylstilbestrol use in oral contraceptives is no longer popular but its indication in post coital contraception is still widely used (FDA drug bulletin, 1973).

7) Other Indications

Other indications where diethylstilbestrol is used include: Postpartum breast engorgement, Duchennes muscular dystrophy, after sexual assault, sickle cell disease, dysfunctional uterine bleeding, failure of ovarian development and acne (17,32,33).

8) Adverse Effects

Adverse side effect may include headache, nausea, vomitting and mild diarrhoea. Recently it has been postulated that long duration therapy may precipitate some complications in some patients, such as voice change, malignant changes, perepheral venous thrombosis, pulmonary embolism, hypertension and stroke (17,30, 32-35). The drug is teratogenic, may induce malformation and malfunction in offsprings of pregnant women who use it.

5. PHARMACOKINETICS

Diethylstilbestrol is readily absorbed from the gastrointestinal tract. The drug is distributed throughout body tissues. It is bound in 50-80% to proteins (34,35). Borozdina et. al. (36) detected residual diethylstilbestrol in meat of animals fed the drug. The compound was detected in the fat, meat, liver and kidneys. After a single subcutaneous injection the drug was completely eliminated after 5 days, in roosters (36), the drug is slowly inactivated in the liver and excreted in urine and faeces, principally as glucuronide (17,35-37) and as the sulphate (37-39). In prostatic cancer patients the level of the hormone was 100 times higher in prostatic tissues than in fat and muscle (20,21). It is natural to anticipate higher concentrations of the hormone in its natural target tissues than the rest of body tissues (17,32,33,39).

6. METHODS OF ANALYSIS

6.1 Identification Tests

The following tests are described in B.P. 1980 (4):-

1) The light absorption, in the range 230 to 350 nm, of a 2-cm layer of a 0.001 per cent w/v solution in absolute enthanol exhibits a maximum only at 241 nm; absorbance at 241 nm, about 1.2.

2) The light absorption, in the range 230 to 450 nm, of the irradiated solution prepared as directed in the assay, exhibits two maxima, at 292 nm and 418 nm.

3) Dissolve 0.5 mg in 0.2 ml of glacial acetic acid, add 1 ml of orthophosphoric acid and heat on a water-bath for three minutes; a deep yellow color is produced which almost disappears on dilution with 3 ml of glacial acetic acid.

The following identification tests are described in USP XX (1) :

1) Prepare an alcoholic solution containing 10 ug of USP diethylstilbestrol RS and diethylstilbestrol respectively in each ml. Determine the absorbance of each solution in the range 230 to 350 nm, using alcohol as the blank. The spectrum of diethylstilbestrol exhibits a maximum and an additional inflection at the same wavelength as that of the solution of USP diethylstilbestrol RS, concomitantly measured, and the absorpitivity of diethylstilbestrol at the wavelength of maximum absorbance does not differ from that of the Reference Standard by more than 3.0%.

2) Prepare the standard preparation as follows: "Dissolve in alcohol a suitable quantity of USP diethylstilbestrol RS, accurately weighed and prepare, by stepwise dilution with alcohol, a solution containing about 20 ug per ml of this solution with an equal volume of dibasic potassium phosphate solution (1 in 55).
Transfer 4 ml of this standard preparation to a stoppered, 1-cm quartz cell, place about 5 cm from a low-pressure, short wave mercury lamp, rated at from 2 to 20 watts, and irradiate for about 5 minutes. Measure the absorption spectrum, in the range of 250 to 450 nm of this yellow solution exhibits inflections only at the same wavelengths as that of the solution obtained after irradiation of the standard preparation.

6.2 Titrimetric Methods

6.21 Volumetric Determination

Stilbestrol dipropionate was determined volumetrically by Gyenes (40) using the bromination method. 72-82 mg in acetic acid (in 20 ml acetic

acid) were treated with 0.1 N $KBrO_3$ (10 ml), H_2SO_4 (1:1, 0.5 ml) and KBr (300 mg in 1 ml of H_2O) in a stoppered flask and left in the dark at $25^o \pm 3^o$ for 80 to 83 min. KI (500 mg in 20 ml H_2O) was added followed by starch solution and titrated against 0.1 N thiosulphate solution. One molecule of the drug consumes 4 equivalents of bromine. However, for stilbestrol the reaction time before titration was 25 minutes. The limit of error for stilbestrol was $\pm$ 0.5%.

Elsayed and Obiakara (41) assayed stilbestrol in tablets and powders by slightly modifying this method and discussed the reaction kinetics in respect to time of addition and bleaching of the reagent. They concluded that the method is suitable for routine work if other phenolic compounds are absent.

6.3 Spectrophotometry

6.31 Colorimetric Methods

British Pharmacopeia 1980(4) described the following procedure:

Dissolve 20 mg in sufficient absolute ethanol to produce 100 ml and dilute 10 ml to 100 ml with the same solvent. To 25 ml of the resulting solution add 25 ml of a solution prepared by dissolving 1 g of anhydrous dipotassium hydrogen orthophosphate in 55 ml of water, transfer a portion of the mixture to a 1-cm closed quartz cell, place the cell 10 cm from a 15-watts, shortwave, ultraviolet lamp, and irradiate for ten minutes. Measure the absorbance of the irradiated solution at the maximum at about 418 nm, and calculate the content of $C_8H_{20}O_2$ from the absorbance obtained by repeating the operation using diethylstilbestrol EPCRS instead of the substance being examined.

United State Pharmacopia XX(1) described the following procedure:

Standard preparation : Dissolve in alcohol a suitable quantity of USP diethylstilbestrol RS, accurately weighed, and prepare, by stepwise

dilution with alcohol, a solution containing about 20 ug per ml. Mix 25 ml of this solution with an equal volume of dibasic potassium phosphate solution (1 in 55).

Assay preparation: Proceed with a suitable quantity, accurately weighed, of diethylstilbestrol as directed under Standard preparation.

Procedure: (Caution - Protect the eyes from direct rays of ultraviolet light throughout this procedure). Transfer 4 ml of the Standard preparation to a stoppered, 1 cm quartz cell, place about 5 cm from a low-pressure, shortwave mercury lamp, rated at from 2 to 20 watts, and irradiate for about 5 minutes. Place the cell in the sample compartment of a suitable spectrophotometer, and measure the absorbance at the wavelength of maximum absorbance at about 418 nm, using water as the blank. Continue irradiation for successive 1 to 3-minute intervals, measuring at 418 nm until the maximum absorbance (about 0.7) has been obtained. If necessary, adjust the geometry of the irradiation apparatus so as to obtain maximum, reproducible absorbance at 418 nm. Similarly, irradiate a 4-ml portion of the assay preparation, recording the absorbance at 418 nm, at successive short intervals until maximum absorbance is obtained. Concomitantly determine the absorbances of the Assay preparation and the Standard preparation in 1-cm cells at 418 nm, using water as the blank, and subtract these values from those for the respective irradiated solutions, to obtain the corrected maximum absorbances. Calculate the quantity, in ug of $C_{18}H_{20}O_2$ in each ml of the Assay preparation by the formula C(Au/As), in which C is the concentration, in ug per ml, of USP diethylstilbestrol RS in the standard preparation, and Au and As are the corrected maximum absorbances of the irradiated Assay preparation and Standard preparation, respectively.

De Almedia Baltazar and Veira de Arbeu compared different methods used in determination of diethylstilbestrol, hexestrol and dienestrol and developed their own method for dienestrol (42).

In the visible range Goodyear et al (43) made diethylstilbestrol to develop a yellow colour after irradiation of an aqueous acetic acid solution of the drug with UV light, and measured the colour absorptiometrically. They also measured the concentration by absorbancy differences of acid and alkaline solutions in the UV range. Cheng and Burroughs used $SbCl_5$ in ethylene chloride to develop a colour with diethylstilbestrol and used a colorimeter with filters at 525 and 430 mu (44). They found that this method is more sensitive than the method of Goodyear et al (43). The yellow colour produced by nitrated derivatives of stilbestrol and dienestrol with alkali had been used by Tokar and Simonyi (45).

The photometric determination of 126 phenolic compounds in water using group-specific reagents i.e. p-nitroaniline, sulfanilic acid, 4-amino-antipyrine and 3-methylbenzothiazoline-2-ylhydrazine, was carried out by Koppe et al (46). In injectable preparations of lipid solvent, the phenolic hormone was extracted with aqueous 0.1 N NaOH and determined spectrophotometrically at 259 nm, while in tablets after dispersion in dil. HCl and extracted by 0.1 N NaOH and measured at the same wavelength. The absorbance obeyed Lambert-Beer's law at concentrations ranging between 2.5 - 12.5 ug/ml. The standard deviation for injectable solutions determinations was 2.06% while for the tablets was 2.52% (47).

6.32 Fluorimetric Methods

Ponder (48) determined diethylstilbestrol in animal feeds (5 ug/kg) florimetrically by converting it to phenanthrenediol derivative. The feed material was extracted with acetone-isopentyl alcohol (1:1) after initial treatment with acetic acid - ethandiol (1:4) mixture. After purification with solvent partitioning, the drug was converted by UV irradiation into the phenenthrenedione which was then oxidized to the diol and determined as previously described by Umberger et al(49). Ponder (50) assayed the drug and its monoglucuronides in beef meat. Vogt determined diethylstilbestrol in the feces and urine of feeder calves (51).

Kolinski et al (52) extracted the drug from enteric-coated and plain-coated tablets, exposed to UV irradiation (pH 6-7) and the reaction product treated with 2% catechol solution in 2 M HCl at 70°C and the resulting 9,10 diethylphenathrene-3,6-diol was determined photometrically at 410 nm with excitation at 335 nm. For 10 determinations at different days, for 1 mg tablets, the coefficient of variation was 0.6% and recoveries were quantitative down to 0.1 mg/tablet.

After using ion-exchange column (Amberlite X AD-2) and (Polycolor column) followed by Li Chrosorb RP-18 column and an on-line reaction system, Verbeke and Vanhee (53) fluorimetrically determined stilbestrol residues in urine and animal tissues. In the on-line reaction system oxidation was achieved at 75°C by SO_2 in alcoholic solution to form a highly fluorescent compound, which, after excitation at 260 nm was measured at 370 nm and they concluded that this method is superior to H.P.L.C. methods.

The following fluorimetric procedure is recommended by the B.P. (4) for the drug tablets:

Procedure

Weigh and powder 20 tablets. To a quantity of powder equivalent to 5 mg of stilbestrol add 50 ml of absolute ethanol, shake for 15 minutes, add sufficient absolute ethanol to produce 100 ml and centrifuge. Dilute 20 ml of the suparnatant liquid to 50 ml with absolute ethanol and to 25 ml of the resulting solution add 25 ml of a solution prepared 1 gram of anhydrous dipotassium hydrogen orthophosphate in 55 ml of water. Transfer a portion of the mixture to 1-cm closed quartz cell, place the cell 10 cm from a 15 watt shortwave, ultraviolet lamp, and irradiate for 10 minutes. Measure the absorbance of the irradiated solution at the maximum at about 418 nm and calculate the content of stilbestrol from the absorbance obtained by repeating the operation with diethylstilbestrol EPCRS.

6.4 Polarography

Direct current and alternating current polarographic responses of some pharmaceuticals, including estogenic compounds, in an aprotic organic solvent system were investigated by Woodson and Smith (54). The application of polarography in stilbestrol analysis was demonstrated by Erb et al (55). Pulse and differential pulse polarography after irradiation of the drug by U.V. was carried out by Kubes (56) to enable determination of the compound as the diketone, and its peak at $^{-}0.8$ V (vs the s.c.e) was measured and the response was rectilinearly related to concentration between 0.75 to 24 uM-stilbestrol.

6.5 Chromatographic Methods

6.51 Paper (PLC) and Thin-Layer Chromatography (TLC):

The earliest of the chromatographic techniques used in estogenic pharmaceutical preparations was paper chromatography (PLC). Paper was impregnated in 5% silicone in cyclohexane, dried at 110°C and spots of 5-50 ug of the oestrogenic compounds were applied and descending chromato-graphy was carried out using the lower layer of the mixture: obtained by mixing 15 ml of water, 85 ml of methanol, 30 ml of benzene and 70 ml of hexane as the moving phase (57). Silica gel G plates and integrating densitometry were combined after column chromatography in the technique used by Jones *et al* (58). The solvent system used for developing the chromatograms was : ethylacetate - light petroleum - anhydrous acetic acid - ethanol (144:27:20:9). Thymoplphthalein indicator was spotted at RF 1 before spraying the plate with Folin-Ciocalteu diluted (1:4) reagent followed by 0.5 N ethanolic KOH, until the indicator spot was permanently blue. Separation of synthetic estrogens from natural estrogens was carried out on silica gel H using chloroform acetic acid (85: 15) (59). Other solvent systems on T.L.C. were tried by Di Domizio and Muscarella (60). Quantitation on silica gel G using two-dimensional chromatography and exposing the chromatograms to 254 nm irradiation following by H_2SO_4 (50%) and

heating at 95°C was carried out by Karkosha (61).

High performance thin layer chromatography (H.P.T.L.C.) using two-dimensional chromatography using hexane - methylene dichloride ethyl acetate (1:2:2) and chloroform - benzene - methanol (36:4:1) was used, for biological extracts (from meat, liver and urine) after purification by gel permeation chromatography, by Smets and Verschaeren (62).

Clarke in his monograph (2) described the following PLC condition.

Sample Preparation

If the sample is urine, it is refluxed with hydrochloride acid, extracted with ether and the ethereal extract is evaporated to dryness. Redissolve in ethanol so that one ml of the ethanolic solution is equivalent to 50 ml of urine. 20 ul of this solution should be applied to chromatogram.

Paper Layer: Whatman No.1, stapled in the form of a cylinder.

Solvent System: Strong ammonia solution : isopropranol : water (1:8:1).

Equilibration: None

Development: Ascending

Location: Pauly's reagent (yellow).

Rf values: Stilbestrol 0.85.

6.52 Gas-Liquid Chromatography (GLC)

McGregor *et al* (63) assayed diethylstilbestrol, extracted from urine, using G.L.C. on a column of 13.5% polyoxyethylene glycol adipate on Chromosorb W at 200°. The hormone was converted to the dimethyl ether. Moretti *et al* (64) derivitized diethylstilbestrol, dienoestrol and hexoestrol as their acetates and used 3% QF-1 liquid phase at

170 °C. The dipropionate of diethylstilbestrol can be determined by direct GLC on 3% JXR on Gas Chrom P at 200°C using stainless steel column. The sample liquid phase was used by Rutherford (65) to determine the cis and trans forms of the estrogenic compound in feeding-stuff pre-mix. The use of a 5% XE-60 on Gas Chrom Z eliminates interference of polyoxyethylene glycol 200 that occurred on 3% JXR column. The electron-capture G.L.C. of the fluoropropionyl diethylstilbestrol or trifluoroacetic anhydride was successful in determination of concentration as low as 1-2 ppb in animal chew or urine (66,67). The use of columns of OV-17 was described by Kohrman and MacGee (68) and Van de Vaart *et al* (69) in determinations carried out in biological and cosmetic materials.

Clarke in his monograph (2) described the following G.L.C. conditions:

Column : 2.5% SE-30 on 80-100 mesh Chromosorb W AWHMDS, 5 ft x 4 mm (i.d.), glass column.

Column Temperature : 225°C.

Carrier Gas : Nitrogen.

Gas Flow Rate : 50 ml/minute.

Detector : Flame ionization detector (F.I.D.).

Hydrogen Flow Rate : 50 ml/minute.

Air Flow Rate : 300 ml/minute.

Retention time 0.78 relative to codeine. At a column temperature of 225°C, the relative retention time to codeine (R.Rt) is 0.78.

6.53 High Resolution Liquid Chromatography (H.P.L.C.) and GLC/HPLC

HPLC was used by several authors in determination of stilbestrol in various formulations and biological materials. The table summarises some of the HPLC (72-79) conditions of techniques used

Table 4 : High-Performance Liquid Chromatography of Diethylstilbestrol.

Column	Mobile phase	Detection	Limit of detection	Ref.
u Bondapak C_{18}	0.1 M CH_3COONH_4 in MeOH – H_2O (3:2) pH 7.1, elution at 1 ml/min.	Electrochemical detection limit cyclic voltammetry at + 0.7V vs the s.e.c.	20 pg	(72)
µ Bondapak C_{18}	75% MeOH	UV (254 nm), resolved cis and trans isomers.	0.2–2 µg/ml	(73)
Li Chrosorb RP-8 or RP-18	Gradient, 10–100% MeOH or RP-8 and 20–100% MeOH for RP-18, 1 ml/min.			(74)
Zorbax SIL	EtOH-Hexane (1:39), 1 ml/min.	UV 240 nm.	0.01 µg.	(75)
RP-2 RP-18 Zorbax CN	Gradient elution:- A: CH_3CN/H_2O(1:9)	Voltammetry with vitreous – carbon and Ag-AgCl (reference) electrodes, Potential sweep rate is 5 mV/Sec.	0.3–0.5 ng in meat (10–50 g)	(76)

Table contd.......

	B: CH_3CN/H_2O(9:1) (25% B at 0 time to 45% B at 5 min). Eluent contains 1 mg of LiCl and 1 mg of $LiClO_4$.			
Li Chrosorb RP-6	MeOH : H_2O (35:13) (25:23)	Vitreous carbon or platinum counter-electrode and a silver-AgCl reference electrode.	1-4 ng/g in meat.	(77)
Li Chrosorb RP-18 or µ Bondapak C_{18}/ Corasil	0.1 N CH_3COONH_4 in in 10-100 MeOH, 2 ml/min.	UV, 254 nm	0.4 ml plasma or urine, 1 g tissue.	(78)
RP-8	50% acetonitrile (pH 3.5) 1.5 ml/min.	In-line photochemical reaction, fluorimetrically at 390 nm (excitation at 280 nm).	low-ppb levels in urine plasma and sera extracts.	(79)

by various authors (66,70-82) while combined GLC/HPLC was tried by others (66,69).

6.54 Combined Gas Chromatography - Mass Spectrometry (GC/MS)

Determinations of anabolic steroids and drugs, including stilbestrol in meat and its products by combined gas chromatography - mass spectrometry (GC/MS) were done by Stan and Abraham (83) and Duerbeck and Bueker (84), in plasma by Gaskell *et al* (85), in urine by Derks *et al* (86), Tuinstra *et al* (87) Diederik *et al* (88) and in baby food samples by Galli *et al* (89). E.I. mass spectrometry was used by most of the authors while Diederik *et al* (88) used negative chemical ionization mass spectrometry using CH_4 OR CH_4 : N_2O (4:1) as chemical ionisation reagents. However, GC-MS technique is the most sensitive technique, other than R.I.A. methods for evaluation of such drugs in biologicals, food and pharmaceuticals and the sensitivity of the technique is 0.1 - 1 ppb.

6.55 Assay by NMR

Al-Badr and Ibrahim (90) developed an NMR spectrometric method for the quantitation of diethylstilbestrol in tablets and ampoules. The method involved comparing the integral of the triplet system of stilbestrol spectrum (positioned at 0.73 σ) to that of the singlet (positioned at 6.25 σ) of maleic acid, as an internal standard. The procedure for the quantitation of the authentic drug, the tablets and ampoules was described.

6.6 Radio Immuno Assay (R.I.A.)

R.I.A. methods have proved to be the most sensitive methods of analysis today. The technique is now a routine in most hospitals and analytical laboratories. Successful R.I.A. of diethylstilbestrol and allied hormones in biological materials and especially in non-ethical use (in meat products) was shown by several authors and the sensitivity of the technique surpasses that of GC/MS method. The sensitivity is in the range 10^{-11} - 10^{-7} (91-96). By this technique it was

determined in blood of feedlot cattle (91), in animal tissues (92,94,96) and in feces (95). Arnstadt (92) used the enzyme immunoassay method by competetive binding stilbestrol and peroxidased labelled stilbestrol to an antibody bonded to DEAE-cellulose. The method is suitable for a range as low as 0.2 pmol (0.05 ng) of stilbestrol.

Gridley *et al* (93) used an immunogen and a second antibody of goat anti-rabbit-O-globulin assay for stilbestrol and its metabolites in boveine liver. Tritium labelled stilbestrol was used and measurement was carried by scintillation counting.

7. METABOLISM

Diethylstilbestrol can be conjugated in the body with H_2SO_4 to a small and variable extent in rats, cats and dogs but not in rabbits (97). Metabolism to monoglucouronide by slices or rat liver was reported (98,99) and also in the everted sacs of the rat intestine (99,100,101). Formations of dihydroxy-diethylstilbestrol and the methoxy derivatives in various species were observed (99,102,103,104). The hormones and its metabolites are excreted via the kidney, liver bile and feces and free stilbestrol presence was observed in feces examined.

McLachlan (105) studied prenatal exposure to diethylstilbestrol in mice and observed equivalent amounts in mother and fetal plasma. The concentration of the drug in fetal genital tract was three-fold higher than that of the plasma.

ACKNOWLEDGEMENT

The authors wish to thank Mr. Altaf H. Naqvi for typing the manuscript.

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FLUOXETINE

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TABLE OF CONTENTS

1. INTRODUCTION

1.1 History

Fluoxetine hydrochloride is a selective serotonin reuptake inhibitor which is clinically effective for the treatment of depression (1). Fluoxetine hydrochloride formulation is marketed as 20 mg (base equivalent) capsules under the proprietary name Prozac®. Fluoxetine and its major metabolite norfluoxetine act as neuronal inhibitors of serotonin reuptake and result in both increased serotonin concentration at the synaptic cleft and autoreceptor stimulation (2-3). Fluoxetine hydrochloride has been shown to have comparable efficacy to tricyclic antidepressants but with fewer anticholinergic side effects (1, 4-6). Fluoxetine hydrochloride has been primarily studied for the treatment of depression, but more recently has been studied for the treatment of bulimia and severe obesity (7-9).

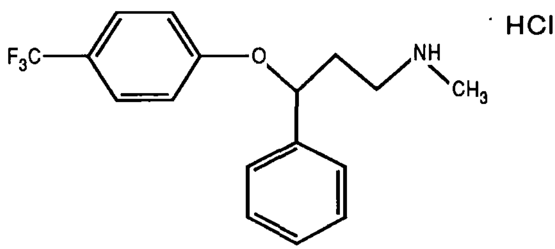
1.2 Name, Formula, Molecular Weight, Structure

Chemical Name: d,l-N-methyl-3-phenyl-3-[(α,α,α-trifluoro-p-tolyl)oxy] propylamine hydrochloride.

Emperical Formula: $C_{17}H_{18}F_3NO{\cdot}HCl$

Molecular Weight: 345.79

Structure:



1.3 Appearance, Color, Odor

Fluoxetine hydrochloride is a white to off-white, crystalline, odorless powder.

2. PHYSICAL PROPERTIES

2.1 Melting Range

The melting range of fluoxetine hydrochloride is 158.4 - 158.9°C

2.2 Simple Solubility Profile

The solubility properties of fluoxetine hydrochloride are listed in Table I. Fluoxetine hydrochloride is freely soluble in methanol and ethanol; soluble in acetonitrile, chloroform, and acetone; slightly soluble in ethyl acetate, dichloromethane, and water (with sonication at pH: 1.2, 4.5, and 7.0). The maximum solubility of fluoxetine obtained in water is 14 mg/mL. Fluoxetine is essentially insoluble in toluene, cyclohexane, and hexane.

Table I. The Solubility Properties of Fluoxetine Hydrochloride.

Solvent	Solubility (mg/mL)
Methanol	> 100
Ethanol	> 100
Acetone	> 33 < 100
Acetonitrile	> 33 < 100
Chloroform	> 33 < 100
Dichloromethane	> 5 < 10
Water	> 1 < 2
(pH 1.2)	> 1 < 2
(pH 4.5)	> 1 < 2
(pH 7.0)	> 1 < 2
Ethyl acetate	> 2 < 2.5
Toluene	> 0.5 < 0.67
Cyclohexane	> 0.5 < 0.67
Hexane	> 0.5 < 0.67

2.3 Elemental Analysis

The elemental analysis data for a typical sample of fluoxetine hydrochloride are listed in Table II.

Table II. Elemental Analysis of Fluoxetine Hydrochloride.

Element	% Calculated	% Found
C	59.05	59.26
H	5.54	5.68
F	16.48	16.52
N	4.05	4.05
O	4.63	4.81
Cl	10.25	9.93

2.4 Infrared Spectrum

The infrared spectrum for fluoxetine hydrochloride in a KBr pellet as obtained on a Mattson Nova Cygni infrared spectrophotometer is illustrated in Figure 1. The major absorption bands for both the infrared and the Raman frequencies and the corresponding assignments are listed in Table III.

Table III. Infrared and Raman Spectral Assignments for Fluoxetine Hydrochloride.

Frequency (cm^{-1})		Correlations/Assignments
Infrared	*Raman*	
3430vb		OH stretch - H_2O in KBr
3085, 3060 3039, 3026 3010	3114, 3087 3064, 3043 3011	Aromatic CH stretches
2986, 2957 2927, 2916	2989, 2969 2962, 2949 2937, 2918	Asymmetric CH_2 and CH_3 stretches
2884, 2861 2838	2893, 2870 2843, 2825	Symmetric CH_2 and CH_3 stretches
2805, 2790 2772, 2730 2638, 2614 2547, 2490 2435	2800, 2769 2746, 2700 2627, 2609 2547, 2495 2458	NH_2^+ - NH stretches and 'combination bands'
1636	1636	NH_2^+ deformations

Table III. (Continued)

Infrared	*Raman*	
1614, 1589 1585, 1516 1495	1615, 1605 1588	Phenyl ring vibrations
1474, 1470	1479, 1470	CH_2 deformations
1455, 1446	1445	Asymmetric CH_3 deformations
1429	1429	Symmetric CH_3 deformations
1329	1326	CF stretches
1314, 1307	1306	Phenyl ring vibrations
1257, 1241	1259, 1244	C-O stretches; aryl/alkyl ether
1182, 1161	1185, 1155	CF stretches; CN stretches secondary amine.
1122,1108	1116	C-O stretches; aryl/alkyl ether
1075, 1069 1028, 1023	1079,1070 1029	Phenyl ring vibrations
999	1003	Ring 'breathing' vibrations mono-substituted phenyl
986, 957 945, 913 903, 847 841, 818	991, 959 948, 916 907, 848 819	Phenyl CH wags
	783	Phenyl ring vibrations Para di-substituted
765, 746 730	768, 747 735	Phenyl ring vibrations

Table III. (Continued)

Infrared	*Raman*	
697		Phenyl ring vibrations mono-substituted
647, 635 622	651, 638 622	Phenyl ring vibrations

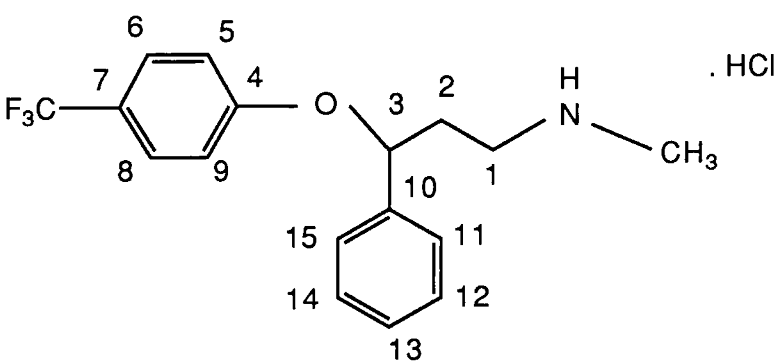
Unassigned:

Infrared: 3225, 3173, 2355, 1970, 1951, 1907, 1897, 1886, 1808, 1010, 878, 858, 805, 671, 594, 563, 520, 512

Raman: 3225, 3202, 3170, 1690, 1391, 1358, 1275, 1211, 1099, 1040, 881, 569, 515, 478, 414, 363, 306, 294, 281, 261, 215

2.5 Nuclear Magnetic Resonance Spectrum

The proton magnetic resonance of fluoxetine hydrochloride is represented in Figure 2. The spectrum was obtained on a General Electric QE 300 MHz NMR using $CDCl_3$ as the sample solvent. Proton chemical shifts were determined from the two-dimensional $^{13}C/^{1}H$ correlation data. The ^{13}C spectrum is shown in Figure 3. Structural assignments are listed in Table IV.



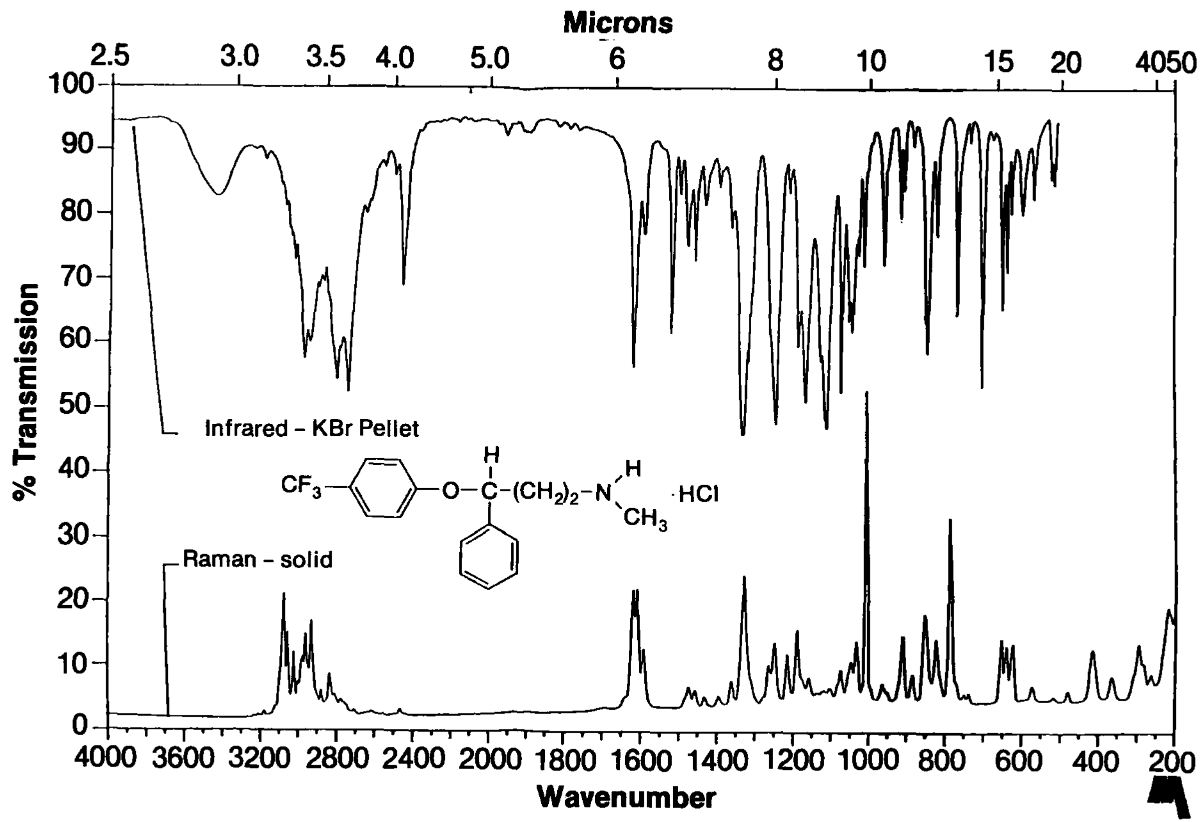


Figure 1. The infrared spectrum of fluoxetine hydrochloride in a KBr pellet.

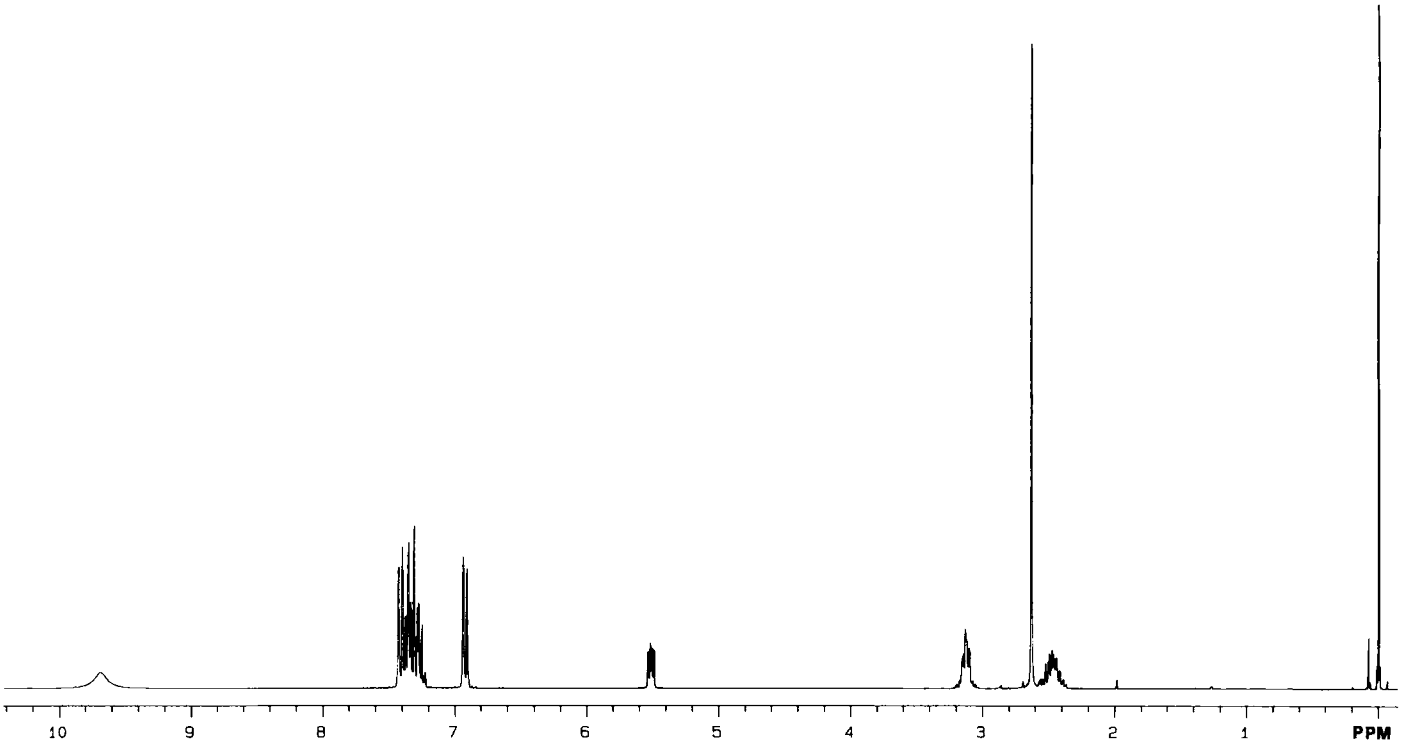


Figure 2. The ^1H NMR spectrum of fluoxetine hydrochloride in $CDCl_3$.

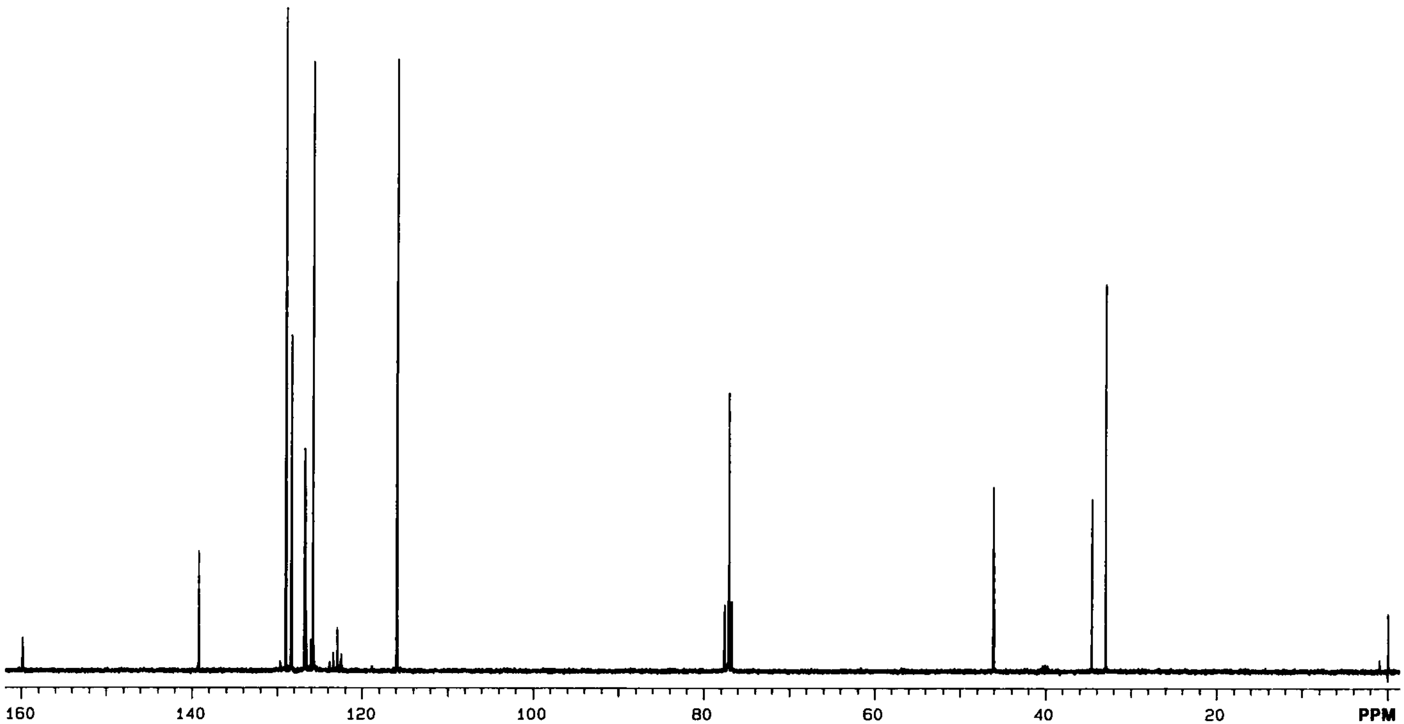


Figure 3. The ^{13}C NMR spectrum of fluoxetine hydrochloride in $CDCl_3$.

Table IV. The NMR Spectral Assignments for Fluoxetine Hydrochloride.

Assignment	^{13}C (Δ)	^{1}H (Δ)
1	46.03	3.12
2	34.49	2.48
3	77.04	5.47
4	139.72	-
5, 9	125.76	6.90
6, 8	126.77	7.39
7	123.33	-
10	159.74	-
11, 15	115.88	7.31
12, 14	128.99	7.27
13	128.38	7.24
CF_3	124.21	-
N-CH_3	32.89	2.62
Exchangeables	-	9.71

2.6 Mass Spectrum

The electron impact (EI) mass spectrum of fluoxetine hydrochloride is illustrated in Figure 4. The spectrum was obtained using a Varian-MAT 731 MS. The EI spectrum for fluoxetine hydrochloride shows a main contribution from the base (MW=309). The EI spectrum is dominated by a major fragmentation from CH_2=NH-CH_3^+ at m/z 44. Several other fragmentations are illustrated in Figure 5 and listed in Table V.

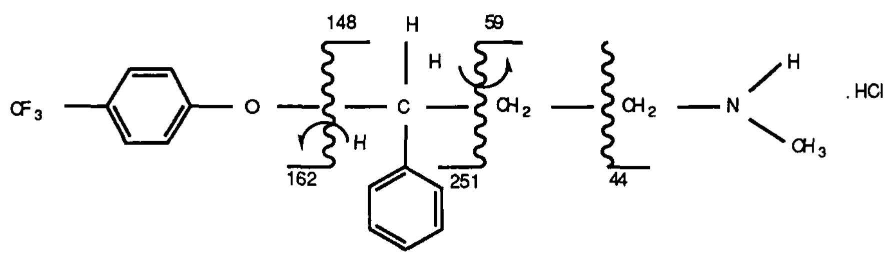


Figure 5. Electron impact fragmentation scheme for fluoxetine hydrochloride.

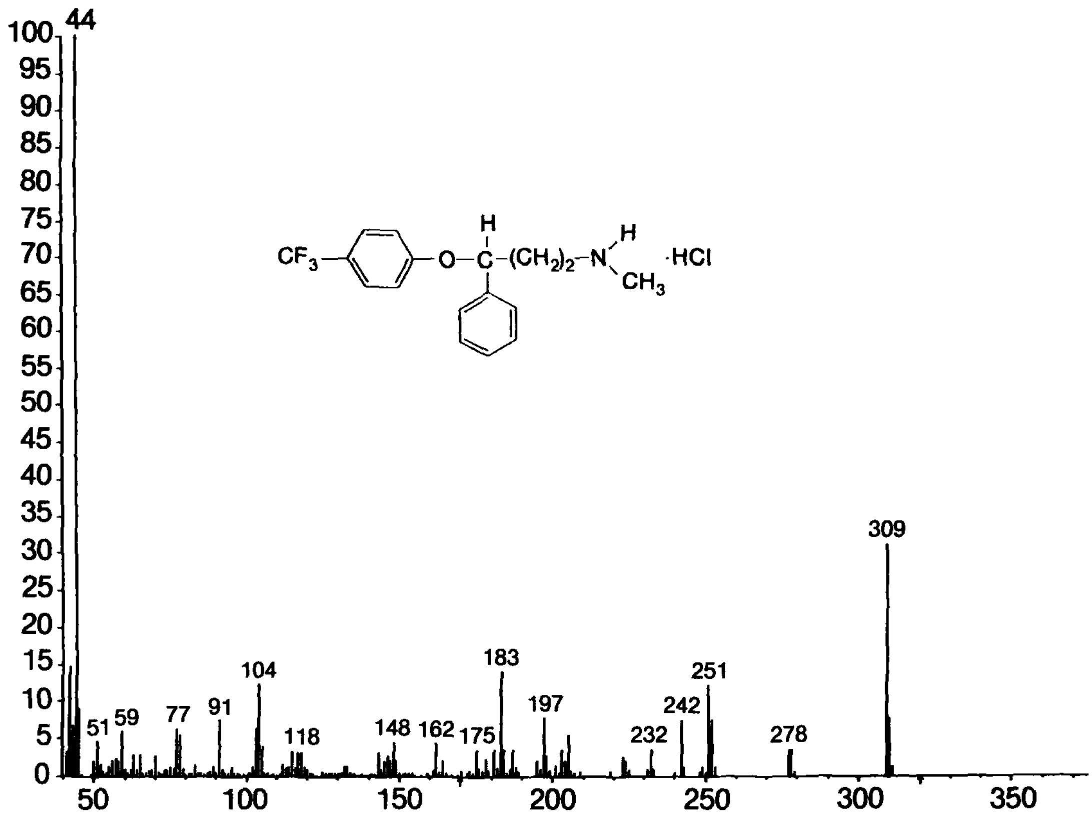


Figure 4. The EI mass spectrum of fluoxetine hydrochloride.

Table V. The Mass Spectrum Fragmentation Assignments for Fluoxetine Hydrochloride.

m/z	Fragment
44	CH_2=NH-CH_3^+
59	$MeNHEt^+$
77	Ph^+
91	Ph-CH_2^+
104	Ph-CH=CH_2^+
148	Ph-CH-CH_2-CH_2-$NHCH_3^+$
162	CF_3-Ph-O^+
251	$[M\text{-}MeNH(CH)_2]^+$

The field desorption (FD) mass spectrum obtained at the optimum anode temperature consists of the molecular ion of the base at m/z 309 and the protonated form of the base at m/z 310. A small fragment peak is present at m/z 104. At higher current emissions, both base and protonated base forms are observed along with cluster ions (base + salt + H) at m/z 655 and (base + 2x salt + H) at m/z 1000. Cluster ion formation is often observed in a FD spectra due to ionization in the condensed phase.

2.7 Ultraviolet Spectrum

The ultraviolet spectrum of fluoxetine hydrochloride in methanol is shown in Figure 6. The ultraviolet absorbance of fluoxetine hydrochloride is due to individual contributions from CF_3-Ph-OR and Ph-R. The maximum absorbance from the fluoronated cresol chromophore occurs at 227 nm with a molecular absorptivity of ε = 12,900. The peak absorbances are listed in Table VI.

Table VI. Peak Absorbances for Fluoxetine Hydrochloride.

Wavelength (nm)	E 1%/1 (cm)	ε
227	372.0	12,900
264	29.2	1,010
268	29.3	1,010
275	21.5	745

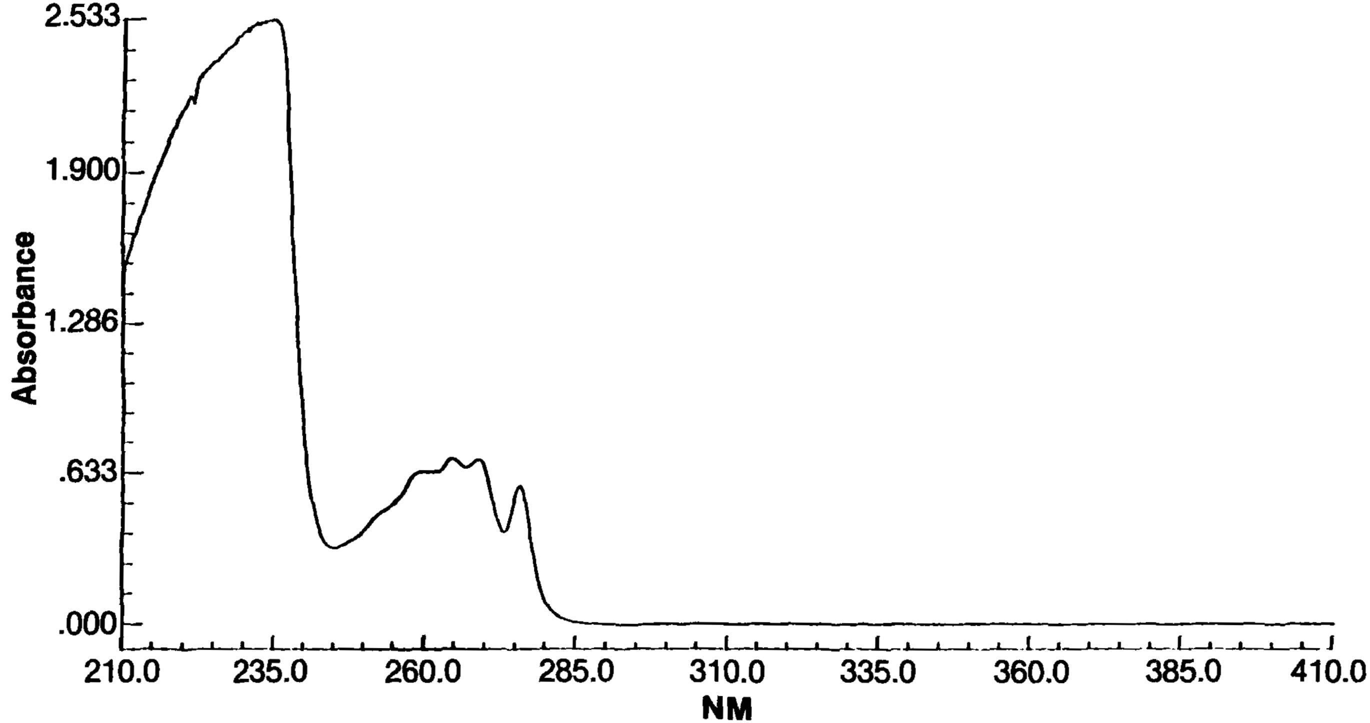


Figure 6. The UV spectrum of fluoxetine hydrochloride in methanol.

2.8 Optical Rotation

The observed rotation for a 10 mg/mL methanol solution of fluoxetine hydrochloride using the sodium D-line on a Perkin-Elmer 241MC Polarimeter was determined to be 0.00° indicating a racemic mixture.

2.9 Differential Thermal Analysis

The DTA thermogram for fluoxetine hydrochloride, at a heating rate of 5°C per minute, shows a sharp endotherm at 161°C indicating a melt. A large exotherm occurs at 241°C and this is attributed to the decomposition of fluoxetine.

2.10 Thermogravimetric Analysis

The TGA thermogram for fluoxetine hydrochloride, at a heating rate of 5°C per minute, shows an initial weight loss at 172°C followed by continuous weight loss indicating decomposition.

2.11 X-Ray Diffraction Analysis

Listed in Table VII are the data from the powder X-ray diffraction for fluoxetine hydrochloride where λ=1.5418, d is the interplanar spacing (A), and I/Io is the relative intensity of the X-ray line. Figure 7 illustrates the X-ray diffraction.

3. SYNTHESIS

The schematic diagram for the synthesis of fluoxetine hydrochloride is outlined in Figure 8.

Synthesis of fluoxetine began with α-[2-(Chloro)ethyl]benzene methanol. A Finkelstein reaction was performed to yield the corresponding iodo derivative. Reaction of this iodide with aqueous methylamine resulted in formation of α-[2-(Methylamino)ethyl]benzene methanol. This material was then reacted with sodium hydride in dimethylacetamide to generate the alkoxide. Addition of 4-fluorobenzotrifluoride led to a facile nucleophilic substitution. Fluoxetine was then reacted with dilute hydrochloric acid to produce the hydrochloride salt (10).

Table VII. X-ray Powder Diffraction Data for Fluoxetine Hydrochloride.

d(A)	I/Io
31.43	0.71
16.14	0.66
8.73	0.07
8.09	0.04
6.71	0.02
6.38	0.60
6.09	0.04
5.39	0.90
4.94	0.03
4.87	0.03
4.79	0.21
4.62	0.01
4.37	0.14
4.27	0.06
4.21	0.01
4.04	1.00
3.92	0.04
3.77	0.15
3.75	0.11
3.59	0.02
3.53	0.06
3.47	0.01
3.36	0.01
3.29	0.01
3.23	0.11
3.19	0.09
3.09	0.21
3.05	0.01
2.99	0.01
2.92	0.01
2.75	0.09
2.57	0.01

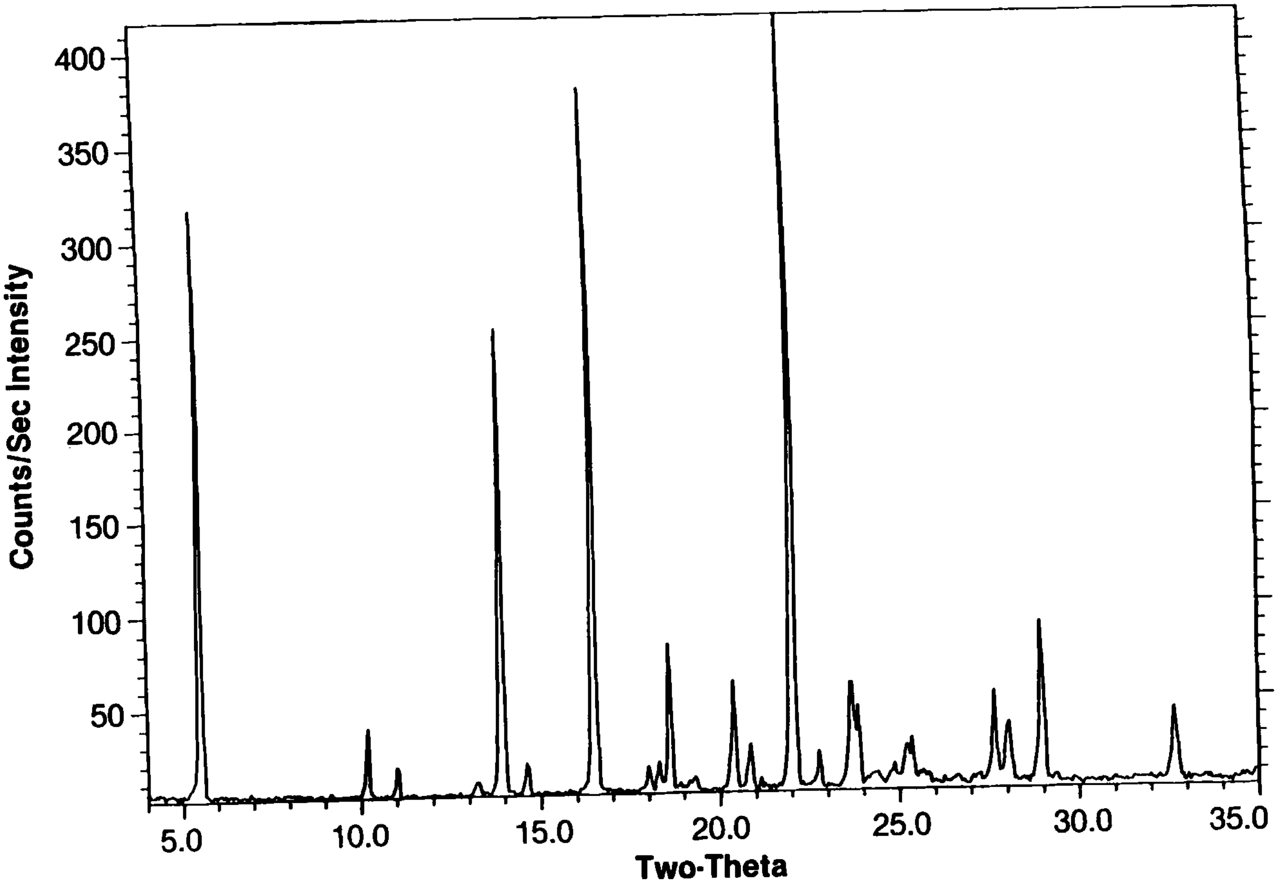


Figure 7. The X-ray diffraction of fluoxetine hydrochloride.

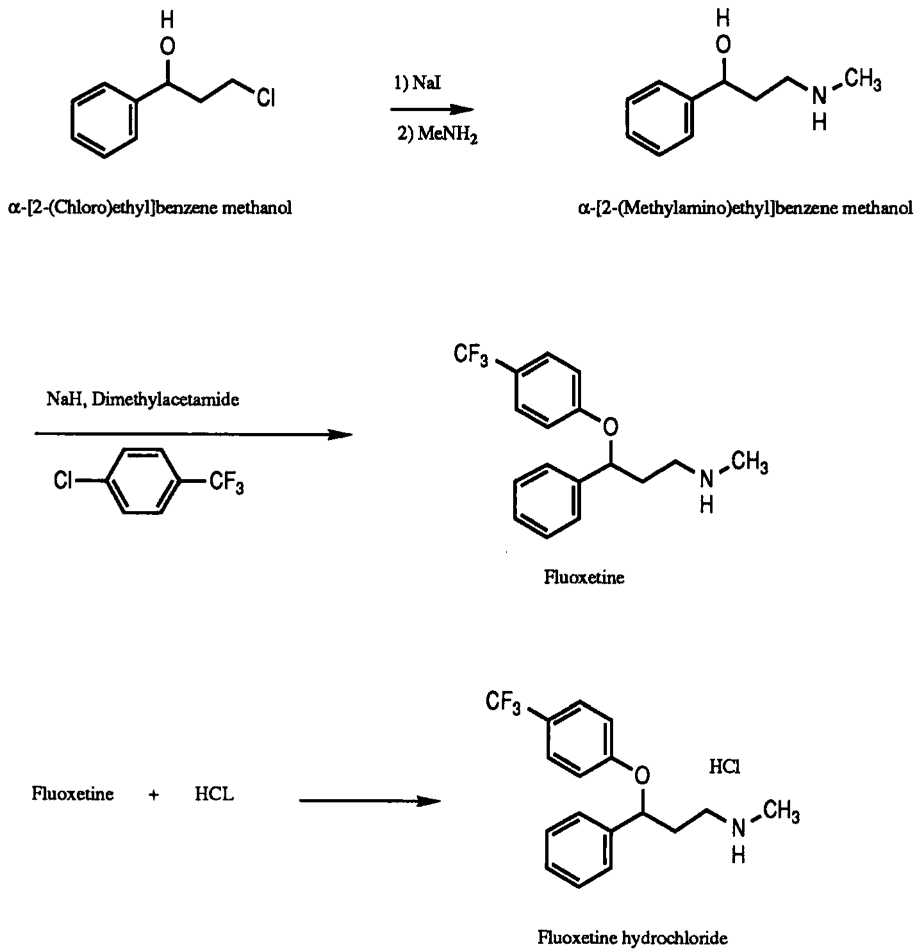


Figure 8. The chemical synthesis of fluoxetine hydrochloride.

4. ANALYTICAL METHODS

4.1 Chromatography

4.11 Thin Layer Chromatography

A thin layer chromatography (TLC) method can be used to determine the identity and purity of fluoxetine hydrochloride. Precoated 20x20 cm silica gel 60 F254 TLC plates are used in combination with a binary solvent system consisting of 90% methanol and 10% concentrated ammonium hydroxide. Visualization is performed by exposing the plate to iodine vapors prior to viewing under short UV light (254 nm). This system will separate the starting material and synthetic intermediates from fluoxetine hydrochloride. These compounds and their respective R_f values are listed in Table VIII.

Table VIII. Thin Layer Chromatography for Fluoxetine Hydrochloride.

Compound	R_f	Identity
Acetophenone	0.84	Starting Material
Benzylmethylamine	0.58	Starting Material
p-Chlorobenzotrifluoride	ND	Starting Material
Paraformadehyde	0.38-0.61 (streak)	Starting Material
ω-(N-methyl-N-benzyl-amino)propiophenone hydrochloride	0.55 to (solvent front)	Intermediate
α-[2-(Methylamino) ethyl]benzene methanol	0.31	Intermediate and Degradation Product
Fluoxetine hydrochloride	0.58	New Drug Substance
N-Methylbenzenepropanamine	0.34	Possible Impurity
p-Trifluoromethylphenol	0.86	Degradation Product

4.12 High Performance Liquid Chromatography

A high performance liquid chromatography (HPLC) method is used for quantitation of fluoxetine hydrochloride at the 0.001% to 0.03% level in rodent feed. The mobile phase consists of 55% methanol/45% water/0.5% diethylamine (water and DEA pH adjusted to 7.4 with phosphoric acid). Chromatographic conditions include a Supelcosil LC8 (150mm x 4.6mm) column combined with a porous silica gel pre-column and packed C8 (pellicular bead) guard column. A flow rate of 1.6 mL/min is used with UV detection at 227 nm. Fluoxetine is extracted from feed using methanol as the extraction solvent. The feed sample and methanol are vigorously shaken for 40 minutes on a wrist-action shaker. A portion of the extraction solution is filtered through fluted filter paper, eluted through an alumina column, and is evaporated to dryness. The residue is reconstituted with mobile phase, and filtered through Whatman #1 filter paper prior to injection.

A reversed-phase HPLC system has been developed to determine fluoxetine in capsule and tablet formulations. The mobile phase consists of 50% acetonitrile/49% water/1% triethylamine (water and triethylamine adjusted to pH 6 with phosphoric acid). A DuPont Zorbax RX column (250 mm x 4.6 mm i.d.) is used for the separation. The flow rate is 1.0 mL/min and UV detection is at 260 nm. The samples are prepared by emptying the contents of a capsule or adding a tablet to a 50 mL volumetric flask, diluting with 40 mL of mobile phase, and sonicating the mixture for five minutes. The sample is cooled and diluted to volume with the mobile phase. This solution is centrifuged for five minutes prior to injecting the supernatant.

A reversed-phase HPLC system can be used for the quantitation of fluoxetine and norfluoxetine (γ-[4-(trifluoromethyl)phenoxy]benzene-propanamine) extracted from serum. The assay is linear for these two compounds over the concentration range of 25-800 ug/mL (11). An alternate reverse phase HPLC method can be used for the determination of these two compounds in plasma by an extraction procedure into hexane (12).

4.13 Gas Chromatography

Due to the reasonable thermal stability and the volatility of fluoxetine, a gas chromatography (GC) method has been used to determine fluoxetine in dosage forms. The chromatographic method was developed on a Hewlett-Packard model 5710 gas chromatograph. A 15 m x 0.53 um x 1.5 um DB-1 Megabore column (J&W Scientific) is used with an oven temperature of 170°C. The injection port temperature is 275°C and the FID detector temperature is 250°C. Helium flow rate is 25.0 mL/min, oxygen flow rate is 300 mL/min, and hydrogen flow rate is 40 mL/min. This procedure involves extraction from basic solution into chloroform. The chloroform extract is filtered through Whatman #1 filter paper prior to analysis.

A GC method is used for the quantitation of related compounds and raw materials of fluoxetine hydrochloride. Optimized conditions are as follows for a Hewlett-Packard model 402 gas chromatograph with a flame ionization detector. The column is 3 ft. x 1/8" i.d. glass packed with 4% OV-225 on 80/100 mesh AWDMCS Chromosorb G. Oven temperature is maintained at 180°C, the injection port temperature is maintained at 245°C, and the detector temperature is 265°C. Gas flow rates are 300 mL/min for oxygen, 60 mL/min for helium, and 40 mL/min for hydrogen. N-pentacosane (0.8 mg/mL) in chloroform is used as an internal standard. All available precursors, intermediates, impurities, and degradation products were analyzed according to this method; the compound names and approximate retention times are listed in Table IX.

A GC method is used for the quantitation of fluoxetine and norfluoxetine in plasma, urine, and tissues. This method requires the formation of the PFB derivative prior to analysis. Conditions were optimized using an HP model 5713 GC with electron capture detection (13). An alternate GC-ECD can be used for the determination of these two compounds in serum (14).

4.2 Spectroscopy

4.21 Infrared Spectroscopy

Fluoxetine hydrochloride is mixed with potassium bromide. The mixture is pressed into a transparent pellet and a spectrum is recorded with a suitable infrared spectrophotometer. This infrared spectrum is used for identity confirmation when it compares favorably to a reference standard spectrum run under similar conditions.

Alternatively, the infrared spectrum for fluoxetine (free base) can be identified after extraction from a basic solution into chloroform. Positive identification is made when the sample spectrum compares favorably to a reference spectrum obtained in the same manner.

4.22 Nuclear Magnetic Resonance

Fluoxetine hydrochloride is added to a nuclear magnetic resonance sample tube and dissolved in deuterated chloroform and a small aliquot of tetramethylsilane. A reference standard spectrum is obtained in the same manner. Positive identification occurs when the chemical shifts and integral values of the sample compare favorably to the reference spectrum.

Table IX. Gas Chromatography for Fluoxetine Hydrochloride.

Compound	Compound Use	Retention (min)
p-Chlorobenzotrifluoride	Starting Material	<0.5
3-Nitro-4-trifluoromethylphenol	Intermediate	<1
2-Nitro-4-trifluoromethylphenol	Intermediate	<1
2-Amino-4-trifluoromethylphenol	Intermediate	3
p-Trifluoromethylphenol	Starting Material, Degradation Product	<1
(3-Chloropropyl)benzene	Starting Material	<1
(1-Bromo-3-chloropropyl)benzene	Intermediate	1.5
α-(2-Chloroethyl)benzyl-α,α,α-trifluoro-*p*-tolylether	Intermediate	5.5
N-Methyl-α-phenylpropylamine	Intermediate	<1
3-Phenylallyl-α,α,α-trifluoro-p-tolyl ether	Impurity	6.5
N-Methyl-α-[3-(trifluoromethyl) phenoxy]benzenepropanamine	Impurity	5
N,N-Dimethyl-α-4-(trifluoromethyl) benzenepropanamine	Impurity	4
(3-Phenylpropyl)(α,α,α-trifluoro-*p*-tolyl)ether	Impurity	3.5
N-Methyl-3-phenyl-2-propen-1-amine	Impurity Degradation Product	0.5
dl-N-Methyl-3-phenyl-3[(α,α,α-trifluoro-*p*-tolyl)oxy]propylamine	Drug Substance	6
α-[2-(Methylamino)ethyl] benzene methanol	Degradation Product Intermediate	2.7

5. STABILITY

5.1 Stability in Bulk

Fluoxetine hydrochloride is a very stable molecule under normal storage conditions. The only known degradation products are α-[2(methylamino) ethyl]benzene methanol and *p*-trifluoromethylphenol. They are formed under acidic stress conditions (3 mg fluoxetine/1 mL 0.1 N HCL refluxed for 48 hours) or when irradiated for five hours with a mercury arc lamp (15).

Fluoxetine hydrochloride is stable as the bulk drug stored at 25°C for five years and at 50°C for two years. No increase in related substances have been observed using these milder storage conditions.

5.2 Stability in Dosage Form

Fluoxetine hydrochloride is marketed as 20 mg (fluoxetine base) capsules. The active ingredient is stable in the starch formulated dosage form stored at 25°C for five years and 40°C/75% relative humidity for two years.

Fluoxetine hydrochloride is stable in a mint syrup formulation (20 mg base/5 mL syrup solution) stored at 25°C for six months. Only a slight increase in related substances was noted at 40°C storage for three months.

5.3 Stability in Solution

Fluoxetine hydrochloride is stable in water even when stored at 65°C or exposed to ultraviolet light for eight weeks. Fluoxetine hydrochloride is also stable in commercially available beverages (Gatorade®, Spea's Farm® Apple Juice, and Ocean-Spray Cran-Grape® Drink) stored at 25°C for two weeks.

6. BIOPHARMACEUTICAL PROFILE

6.1 Pharmacokinetics

In human volunteers, fluoxetine is readily and completely absorbed from the gastrointestinal tract with peak serum levels occurring 6-8 hours after oral dosing with capsules (16, 17). Fluoxetine and its major metabolite norfluoxetine are distributed in the tissues, predominantly the lung, and gradually released. The elimination half life of fluoxetine is 2-3 days and that of norfluoxetine is 7-9 days (17). Maximal central nervous system efficacy has been shown to be 8-10 hours post dosing (18).

6.2 Drug Metabolism

The main metabolite of fluoxetine is norfluoxetine, an active metabolite with similar physiological activity as its parent compound. Metabolism occurs in the liver by N-demethylation (17, 19). Studies in animals have shown that fluoxetine is also metabolized into *p*-trifluoromethylphenol by O-dealkylation (20). Other known metabolites include glucuronides of both fluoxetine and norfluoxetine.

After oral dosing with C-14 labeled drug, 60% of the activity was recoverd in the urine over a 5 week period (2.5-5.0% was recovered as unchanged drug, 10% being norfluoxetine, 5.2% fluoxetine glucuronide, and 9.5% norfluoxetine glucuronide). An additional 16% of the radio-labeled material was recovered in feces (17).

6.3 Toxicity

Acute toxicity of fluoxetine was determined in mice, rats, dogs, and monkeys. LD_{50} values are listed below in Table X.

Table X. LD_{50} Data for Fluoxetine Hydrochloride.

Species	Route	LD_{50} (mg/kg)
Mouse	Oral	248
Mouse	I.V.	45
Rat	Oral	466
Rat	I.V.	35
Dog	Oral	ND*
Monkey	Oral	ND*

* Not Determined (Dog LD_0 > 100; Monkey LD_0 > 50)

Phospholipids were increased in some tissues of rats and dogs, however, phospholipidosis was found to be reversible after cessation of fluoxetine dosing. In rodents, elevated doses of fluoxetine resulted in the following toxicity signs: increased salivation, tremors, ataxia, leg weakness, and chronic convulsions. In dogs, vomiting, mydriasis, tremors, and anorexia were observed. In monkeys, vomiting was the only indication of toxicity. Toxicity studies in animals administered chronic dosing of fluoxetine indicate that the drug was not a mutagen, carcinogen, or teratogen, and did not impair reproductive capabilities (20).

Side effects noted after dosing with fluoxetine in human volunteers include: nausea, nervousness, insomnia, headache, tremor, anxiety, and drowsiness. Uncommon side effects include: psychosis, hallucinations, ataxia, dizziness, sensation disturbances, and asthenia (21).

8. ACKNOWLEDGEMENTS

The following people are sincerely thanked for providing valuable information for specific sections of this chapter:

L. G. Tensmeyer for the infrared assignments.
S. R. Maple and J. W. Paschal for the NMR interpretation.
G. G. Cooke and J. M. Gilliam for the mass spectral interpretation.
G. A. Stephenson for the X-ray crystal interpretation.
B. G. Jackson for the synthesis.
A. Hunt for the UV interpretation.
K. A. McCune for spectral plots.
D. R. Long for the thermal analysis data.
P. N. Anderson and T. J. Wozniak for chapter review.

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ANALYTICAL PROFILE OF FOLIC ACID

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

a. N-(p-{(2-amino-4-hydroxypiramido [4,4-b]pyrazin-6-yl) methylamino] benzyoyl)-glutamic acid (1).

b. 4-2-amino-4-hydroxypteridin-6-yl)methylamino-benzoyl-L-glutamic acid (2).

c. N-[4-[{(2-Amino-1,4-dihydro-4-oxo-6-petridinyl) methyl] amino] benzoyl}-L-glutamic acid (1,3).

d. N-[4-(2-Amino-4-hydroxy-ptridine-6-ylmethyl-amino) benzoyl]-L(+)-glutamic acid (4).

e. 4-(2-Amino-4-hydroxy-6-ptridinyl)methylamino-benzoyl-L-glutamic acid (5).

f. N-[p-[{(2-amino-4-hydroxy-6-pteridinylmethyl} amino} benzyoyl]-glutamic acid (1, 3, 6).

1.1.2 Generic Names

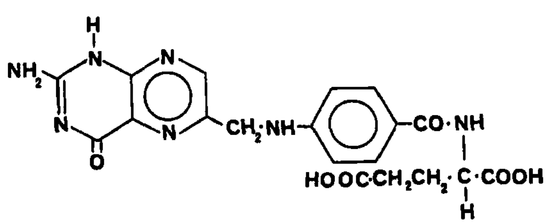
Acidum, cytofol, folsavre, folacin, foldine, folaemin, foliamin, folicet, folipac, foletts, folsan, folvile, folicum, folinsyre, incafolic, liver lactobacillus casei factor, mallafol, nifolin, nifola acid, pteroylglutamic acid, PGA vitamin Bc, vitamin M.

1.2 Formulae

1.2.1 Empirical

$C_{19}H_{19}N_7O_6$

1.2.2 Structural



1.2.3 CAS Registry Number

59-30-3

1.2.4 Optical Rotation (1)

$[\alpha]_{D}^{25}$ + 23 (C = 0.5 in 0.IN NaOH)

1.2.5 Wiswesser Line Notation (7)

T66 BN DN GN JNJ CZ EQ H1 MR DVMYVQ2V Q

1.3 Molecular Weight

441.40

1.4 Elemental Composition

C, 51.70% H, 4.34% N, 22.22% O, 21.75%

1.5 Appearance, Color, Odor and Taste

A yellowish to orange, crystalline powder, almost odourless (2), tasteless microcrystalline powder containing 5 to 8.5% of H_2O (4).

2. Physical Properties

2.1 Melting Range

No m.p. darkens and chars from 250°C (1).

2.2 Solubility

Very slightly soluble in cold water (0.0016 mg/ml at 25°C) soluble to about 1% in boiling water. Slightly soluble in methanol, appreciably less soluble in ethanol and butanol. Insoluble in acetone, chloroform, ether, benzene. Relatively soluble in acetic acid, phenol, pyridine, solutions of alkali hydroxides and carbonates (1). It is soluble in hot diluted HCl and H_2SO_4 (3). Soluble in HCl and H_2SO_4 yeilding very pale yellow solutions (3).

A solution in 0-1M NaoH is dextrorotatory (4). Folic acid injection is a sterile solution of folic acid in water with the aid of sodium hydroxide or sodium carbonate, the addition of sod. hydroxide or sod carbonate to the injection result in formation of sodium folate which is the soluble sodium salt of folic acid (8). Commercially available folic acid injection is clear, yellow to orange in color (8).

2.3 Stability

A solution of folic acid 1 mg per ml, in a vehicle of purified water preserved with hydroxy benzoates and adjusted pH 8-8.5 with NaOH, had little loss of potency when stored at 25°C for 8 weeks (4). Folic acid is labile to acid, 70-100% of the activity being destroyed on autoclaving at pH 1 (9,10). It becomes progressively more stable as the pH increases and is relatively stable to heat within the pH range 4 to 12. At pH 6.8, for instance, solutions can be sterilized by heating for thirty minutes without loss of potency. Folic acid is partially inactivated by lead and mercury salts (9) and by treatment with sulphite (10). Aeration at pH 1 also causes partial inactivation. In pure solutions, it is rapidly inactivated by light with the formation of P-amino-benzyoylglutamic acid (11) and 2-amino-4-hydroxy-6-formylpteridine (12), the later is converted first in to the corresponding acid and then into 2-amino-4-hydroxypteridine.

2.4 pH

A suspension of 1 gm folic acid in 10 ml H_2O has a pH of 4.0-4.8. Aqueous solutions prepared with sodium bicarbonate have a pH between 6.5 and 6.8 (1).

2.5 Occurrence

Folic acid is widely distributed in plants, animals and micro-organisms. Drug is present free or combined with one or more additional molecules of L(+)-glutamic acid in liver, kidney, mushrooms, spinach, yeast, green leaves, grasses (1). Drug is present (13) in liver, kidney, yeast mushrooms, grass and other green leaves and also present (14) in many micro-organism, the highest yields (0.25 to 1.67 mg. per

ml.) being obtained from *B. subtilis*, *B. Vulgatus*, *serratia marcescens* and a Gram-negative bacillus from chick intestine.

2.6 X-Ray Powder Diffraction (15)

The x-ray diffraction pattern of folic acid (Fig. 1) was determined on a Philips x-ray diffraction spectrogoniometer equipped with PW 1730/10 generator. Radiation was provided by Copper target (Cu anode 2000W, = 1.5418 A) and high intensity x-ray tube operated at 40 KV and 35 MA. Divergence slit and the receiving slit was 1° and 0.1° respectively. The unit was equipped with Philips PM 8210 printing recorder and digital printer. The x-ray diffraction pattern is listed in Table (1).

The crystal structure of folic acid has also been reported by Donald Mastropaolo et al. (16). Fig. (2a) shows a line drawing of the molecule with the numbering scheme which is used in the description of the structure. A stereoscopic view of the molecule (Fig. 2b) shows folic acid to be an extended conformation. The substituted pterin and p-aminobenzoyl groups are planner to within 0.06 and 0.03 Å, respectively and the dihedral angle between their plannes is 27°. The two substituent atoms N(2) and C(9) are 0.11 Å from the least-squares plane of the pteridine ring system; atoms N(18) and C(19) are 0.01 and 0.03 Å, respectively, out of the plane of the p-aminobenzoyl group. The values of the following selected torsion angles indicate the extended nature of the conformation.

N(5)-C(6)-C(9)-N(10)	31°
C(6)-C(9)-N(10)-C(11)	180°
C(13)-C(14)-C(17)-N(18)	175°
C(14)-C(17)-N(18)-C(19)	-178°

The observed C(4)-O(4) bond distance is 1.23 ± 0.03 Å; this value plus location of the hydrogen attached to N(3) clearly establishes the keto rather than the enol form of the folic acid molecule in the crystal.

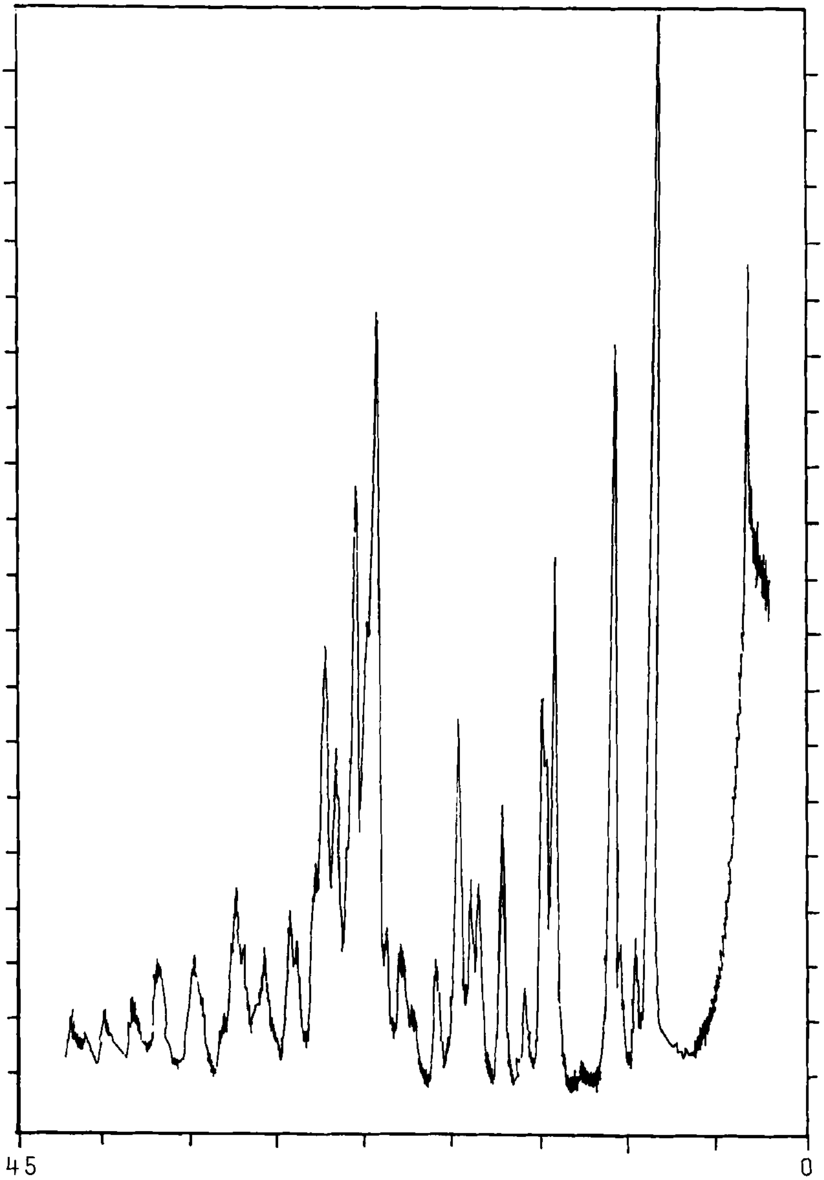
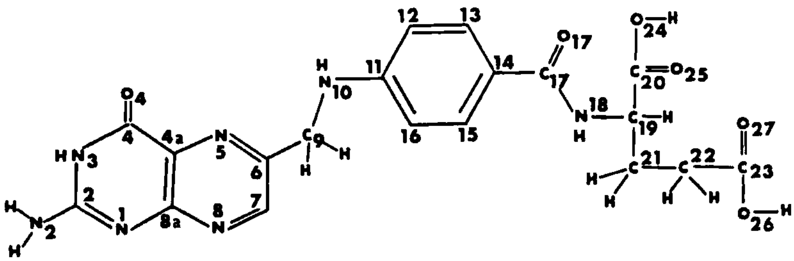


Fig. 1: X-Ray Powder Diffraction of Folic Acid

Fig. (2a): Folic Acid Chemical Structure and Atom Numbering Scheme



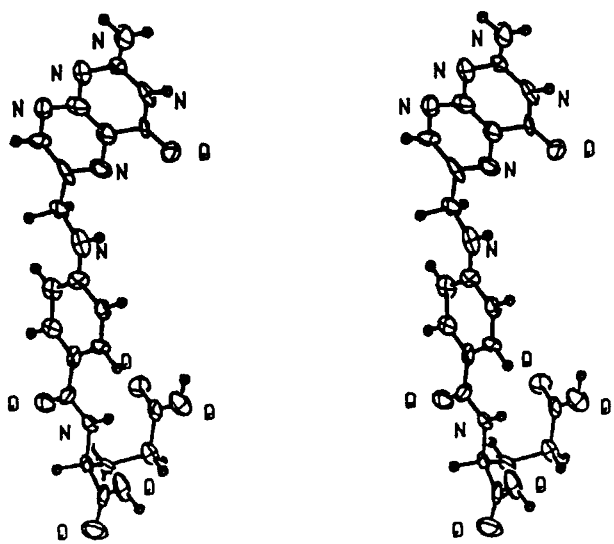


Fig. (2b): Sterioscopic Drawing of the Folic Acid molecular structure

Table (1): Characteristic lines of the X-ray powder diffraction of folic acid

2θ	dÅ	I/Io X 100
5.358	16.4919	55.824
10.811	8.1833	100
11.465	7.7180	12.161
12.361	7.1604	12.161
12.979	6.8209	50.451
14.489	6.1133	3.928
16.304	5.4364	37.582
16.804	5.2760	24.157
17.774	4.9900	9.542
18.158	4.8854	5.930
19.203	4.6218	20.815
20.419	4.3493	16.405
20.883	4.2537	16.405
21.664	4.1020	26.866
22.780	3.9036	10.565
24.053	3.6998	6.803
24.739	3.5987	10.565
25.694	3.4670	13.260
26.506	3.3626	34.527
26.964	3.3066	31.607
27.675	3.2232	40.231
28.663	3.1143	24.668
29.374	3.0405	30.930
29.809	2.9972	16.194
30.778	2.9050	11.920
31.169	2.8695	13.696
32.641	2.7433	11.679
33.898	2.6444	12.086
34.373	2.6090	15.141
34.971	2.5657	9.043
36.288	2.4756	8.865
36.703	2.4485	11.258
38.573	2.3340	9.000
40.010	2.2534	8.067
40.215	2.2424	8.789
40.940	2.2044	5.945
41.791	2.1614	7.706
43.559	2.0777	7.676

2.7 Spectral Properties

2.7.1 Ultraviolet Spectrum (15)

Ultraviolet spectrum of folic acid in 0.1 N NaOH was scanned from 200 to 400 nm using LKB 4054 LKB UV/vis spectrophotometer (Fig. 3). It exhibited the following UV data (Table 2).

Table (2): UV Characteristics of folic acid

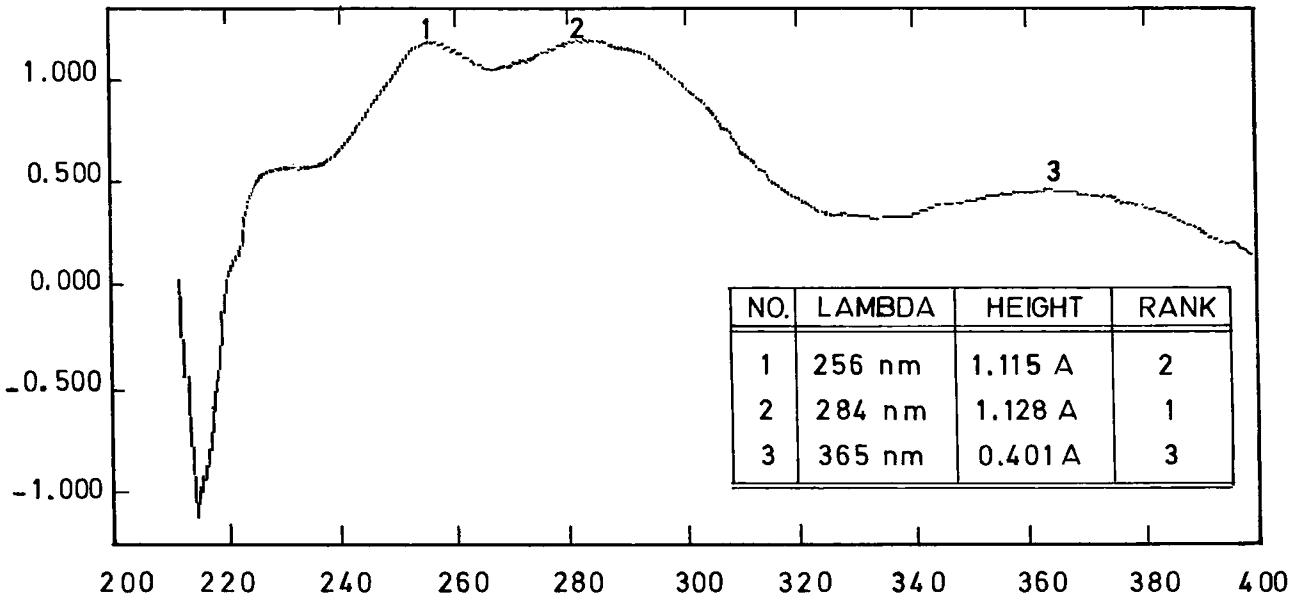
max nm	ϵ (molar absorptivity)
256	49171.5
284	49744.8
365	17684.1

2.7.2 Infrared Spectrum

The IR spectrum of folic acid as KBr-disc was recorded on a Perkin Elmer 580 B Infrared spectrophotometer to which an infrared data station is attached (Fig. 4). The spectral band assignments are listed in Table 3.

Table (3): Values of infrared absorption

Frequency cm^{-1}	Type of vibration	Assignment
3520-3200	NH	- CONH group
3000-2500	CH	- CH_2 group
1689	C = O	- COOH group
1600	C = O	- CONH
1474	CH	- CH_2 group
1330	CH	- CH_2 group



NO.	LAMBDA	HEIGHT	RANK
1	256 nm	1.115 A	2
2	284 nm	1.128 A	1
3	365 nm	0.401 A	3

Fig. 3: UV Spectrum of Folic Acid in 0.1 N NaOH

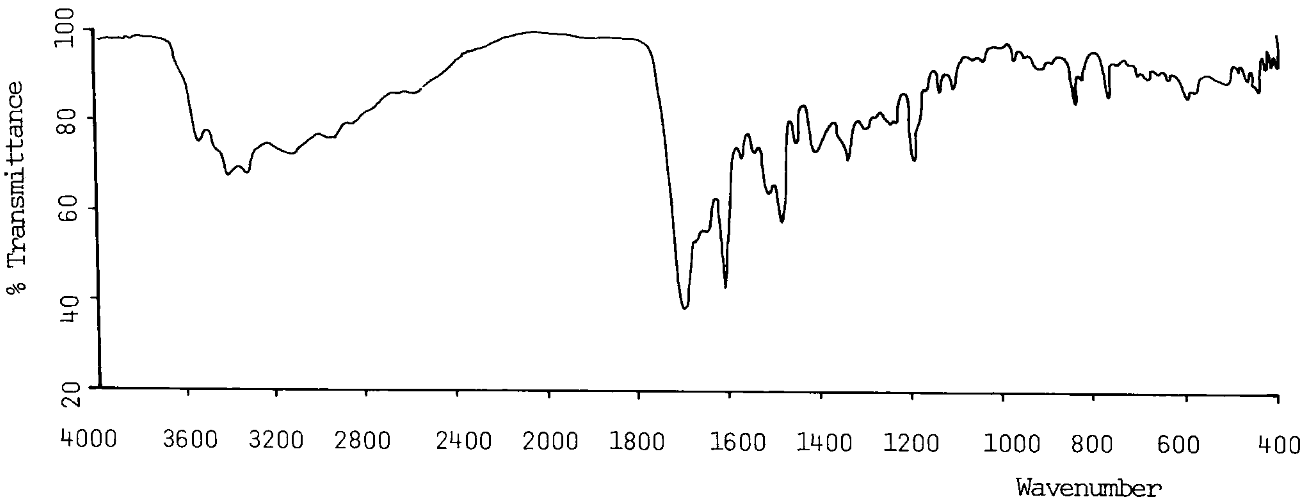


Fig. 4: I.R Spectrum of Folic Acid as KBr disc

2.7.3 Nuclear Magnetic Resonance Spectra

2.7.3.1 PMR Spectra

The PMR spectra of folic acid was recorded on a Varian T60A, 60 MHz NMR spectrometer using TMS as an internal reference. The spectra are shown in Fig. (5a and 5b).

2.7.3.2 ^{13}C NMR Spectra

The ^{13}C NMR spectra of folic acid in DMSO-d_6 is recorded on a Joel FX-100 NMR spectrometer and is presented in Fig. (6).

2.7.4 Mass Spectrum

Secondary ion mass spectra of folic acid (17) is presented in Fig. (7). The measurements were performed by a modified Leybold-Haraeus SIMS apparatus with a Blazer, quadrupole mass filter QMG 1023. The dried samples were introduced into the vacuum chamber via a rod, passing through a lock system. For secondary ion emission the sample was bombarded by a scanned 3-KeVAr^+ ion beam with a current density of 10^{-2} A/0.1 cm^2. For thermal investigations the Ag foils covered with the organic substances were introduced in to the mass spectrometer and directly heated by an electric current. The temperature was determined by a Ni-Cr-Ni element at the lower side of the 0.2 mm Ag foil. Before organic substances were deposited, the Ag foils were heated for 30 min at 400°C under atmospheric conditions. Some characteristic fragment ions, resulting from predictable bond cleavages is mentioned on the spectra (Fig. 7).

3. Synthesis (6)

Folic acid may be synthesized by the following method.

When 2,3. Dibromopropionaldehyde, dissolved in water-miscible organic solvent (alcohol, dioxane) is added to a solution of equal molecular quantities of 2,4,5-triamino-6-hydroxypyrimidine and p-aminobenzoyl glutamic acid, maintaining a pH of about 4 by the controlled addition of alkali as the reaction progresses.

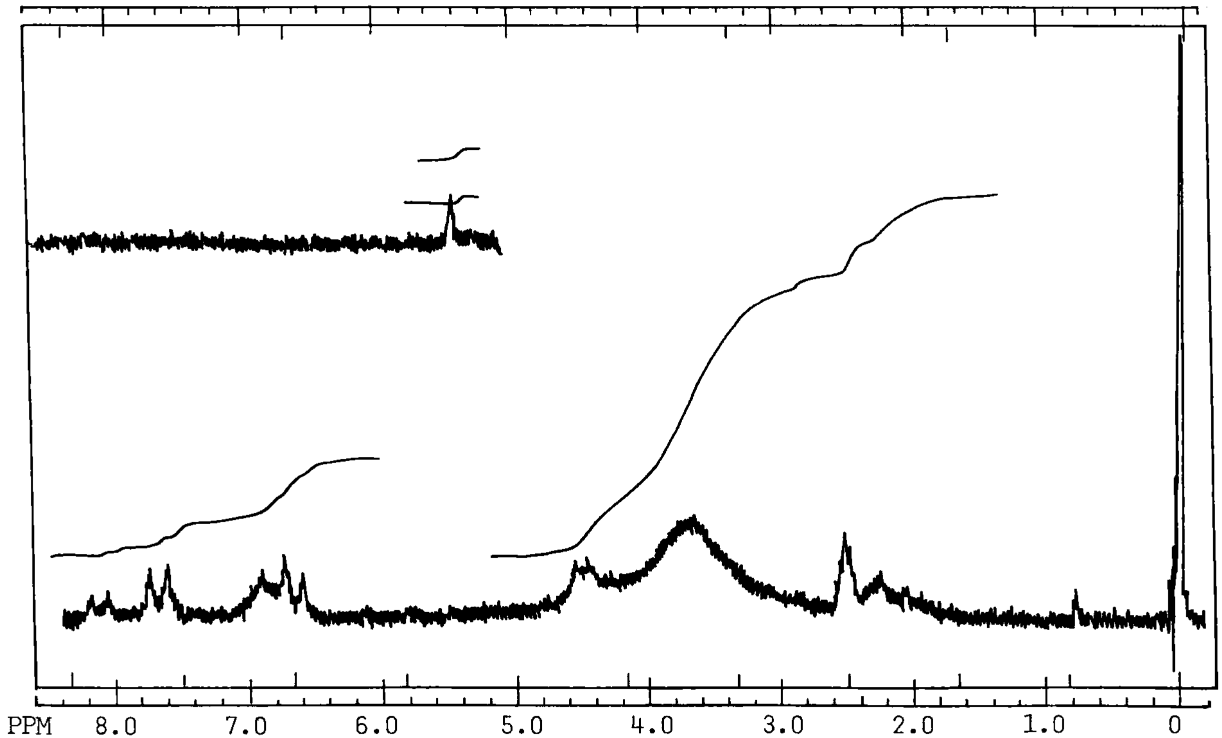


Fig. 5(a): PMR Spectrum of Folic Acid

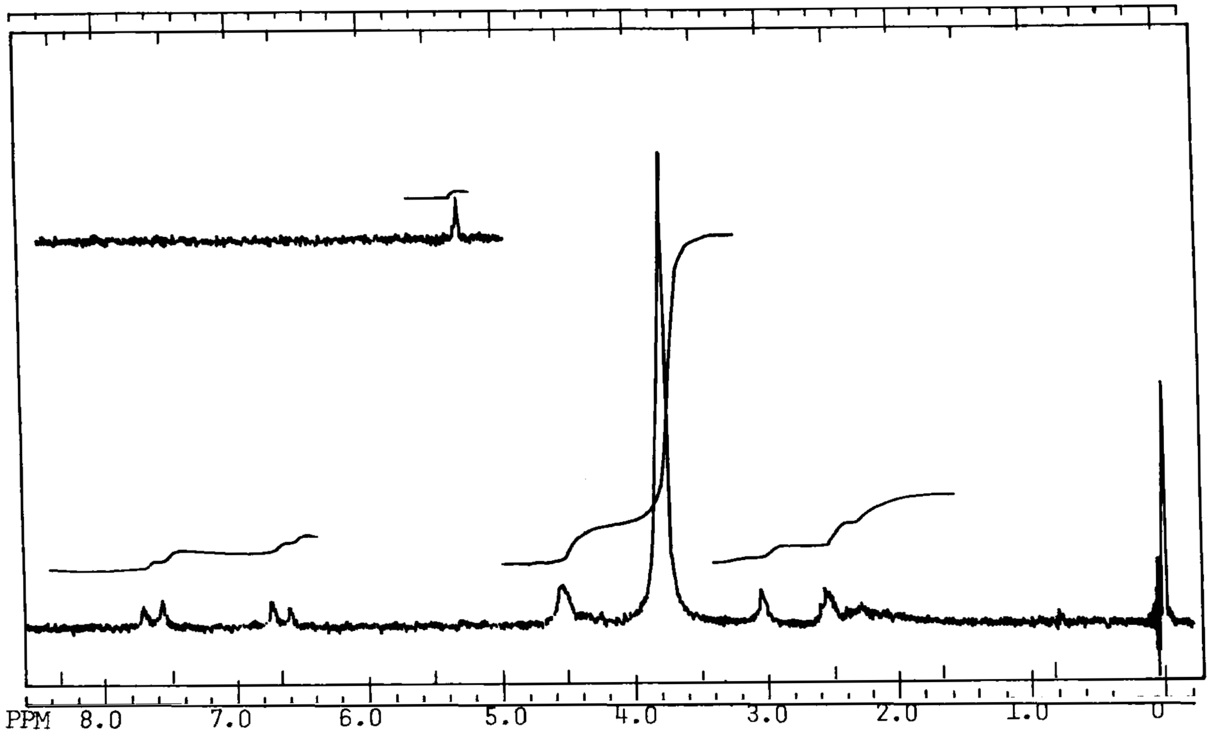


Fig. 5(b): PMR Spectrum of Folic Acid (D_2O exchange)

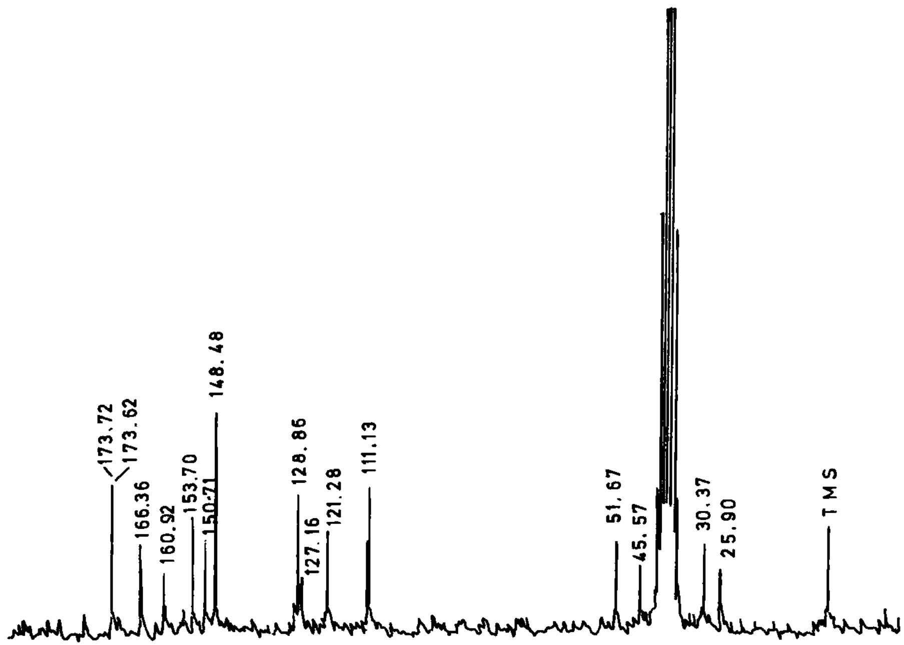


Fig. 6: ^{13}C-NMR Spectrum of Folic Acid in DMSO-d_6

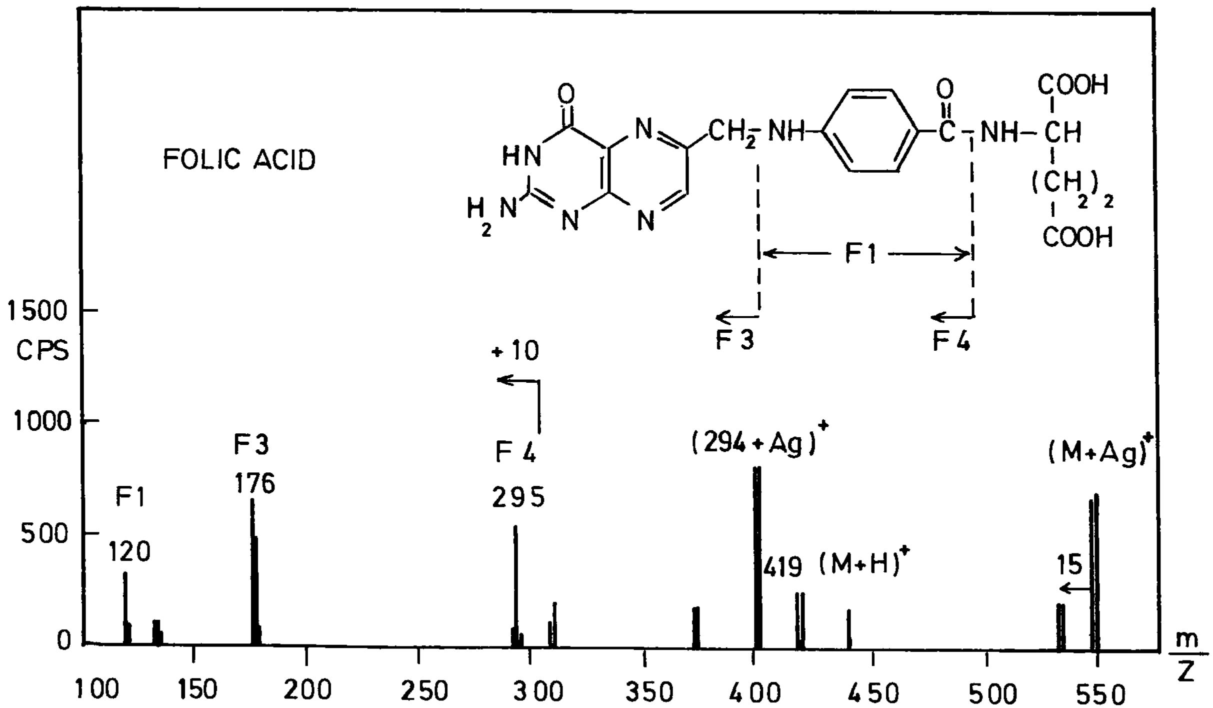
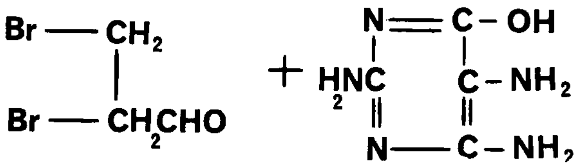


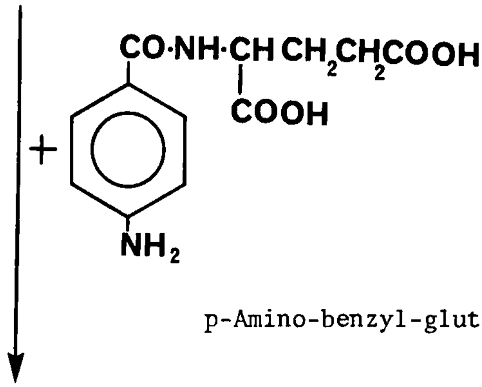
Fig. 7: Secondary ion mass Spectrum of Folic Acid

Scheme: (synthesis)

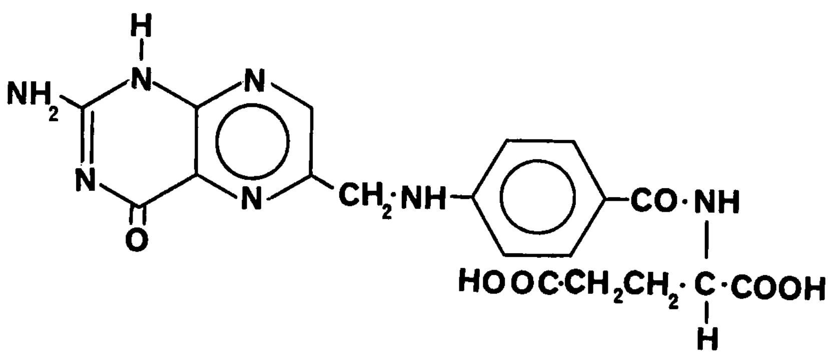


2,3,di-bromo propionaldehyde

2,4,5,triamino-6-hydroxy-pyrimidine



p-Amino-benzyl-glutamic acid



Folic acid

4. Metabolism

Folic acid is absorbed rapidly from the GI tract following oral administration, the vitamin is absorbed mainly from the proximal portion of the small intestine. Naturally occurring folate poly-glutamates are enzymatically hydrolysed in the GI tract to monoglutamate forms of folic acid prior to absorption. Following oral administration, peak folate activity in blood occurs within 30-60 minutes. Normal serum conc.have been reported to range from 0.005-0.015 µg/ml. In general, serum folate conc. below 0.005 µg/ml indicate folate deficiency and conc. below 0.002 µg/ml usually result in megaloblastic anemia. Tetrahydrofolic acid and its derivatives are distributed into all body tissues. The liver contains about one-half of total body folate stores. Folate is actively concentrated in CSF, and normal CSF concentrations are reported to be about 0.016-0.021 µg/ml. Normal erythrocyte folate concentrations range from 0.175-0.316 µg/ml. Folic acid is distributed into milk. Following absorption of 1 mg or less folic acid is largely reduced and methylated in the liver to N^5-methyltetrahydrofolic acid, which is the main transport and storage form of folate in the body. Larger doses of folic acid may escape metabolism by the liver and appear in the blood mainly as folic acid.

Following oral administration of single 0.1-0.2 mg doses of folic acid in healthy adults, only a trace amount of the drug appears in the urine. Following administration of large doses,the renal tubular re-absorption maximum is exceeded,and excess folate is excreted unchanged in urine. After doses of about 2.5-5 mg, about 50% of a dose is excreted in urine and after a 15 mg doses, up to 90% may be recovered in urine. Small amounts of orally administered folic acid have been re-covered from feces. About 0.05 mg per day of normal body folate stores is lost by a combination of urinary and fecal excretion and oxidative cleavage of the molecule (8).

According to Steinkamp et al. (18) normal subject excretes in urine 2 to 4ug per day of pteroylglutamic acid, as estimated microbiologically with S. faecalis R. Following oral administration of a test dose, 15

to 76% with an average of 28.5% was recovered in urine.

The amount of drug excreted increased with the amount administered, being as much as 50% with a 5 mg oral dose, nearly all was excreted within 6 hours. The di- and tri-glutamate, but not the heptaglutamate, were converted pteroylglutamic acid.

The pernicious anaemia patient, receiving 0.85 mg of synthetic folic acid daily, about 15% excreted in urine. Following the intramuscular injection of 30 mg of conjugate, no increase occured in the amount of folic acid excreted but the injection of further 11.mg resulted in the excretion of 4.1 mg in the following 48 hours. (19).

B.C. Johnson (20) studied that human excrete folic acid in sweat but the amount may be 5 or 5-fold the amount eliminated per hour in the urine under conditions of profuse sweating.

Following the IV injection of pteroylglutamic acid an encores in blood concentation took place which reached maximum 2 hours later (21). G. Tonnies (22) studied that when triglutamate injected intramuscularly, two third of the amount remaining in the blood stream two hours later was present in monoglutamate form. The blood of many animals incuding man, contains folic acid conjugate capable of releasing pteroylglutamic acid from the heptaglutamate (23).

5. Deficiency and Uses

Deficiency of folic acid leads to megaloblastic anaemia. Deficiency may result from a diminished intake, as in malnutrition from malabsorption, or from the concomitant use of anticonvulsants or dihydrofolate reductase inhibitors, such as pyrimethamine,trimethoprim, or metho-trexate. Folic acid does not correct folate deficiency due to dihydrofolate reductase inhibitors, calcium folinate is used for this purpose (4).

Megaloblastic and macrocytic anaemia resulting from folate deficiency is usually indicated in the treatment of nutritional macrocytic anemia,

megaloblastic anemia of pregnancy, infancy and childhood, and megaloblastic anemia associated with primary liver disease, alcoholism and alcohlic-cirrhosis intestinal structures anastomoses, or sprue. Folate deficiency may also result from increased loss of folate secondry to renal dialysis or the administration of some drugs as phenytoin, primidone,barbiturates, oral contrarceptives, or salicyl azosulfapyridine.

Folic acid is not effective in the treatment of normocytic, refractory, or aplastic anemias or when used alone,in the treatment of pernicious anemia. Folic acid antago-nistics (eg. methotrexate, pyrimethamine, trimethoprim) inhibit folic acid reductases and prevent the formation of folic acid. Therefore, folic acid is not effective as an antidote following overdosage of these drugs, and leucovorin calcium must be used.

Although prophylactic adminsitration of folic acid is not required in msot individuals, suplemental folic acid may be required to prevent deficiency of the vitamin in patients with conditions that increase folic acid requirements such as pregnancy, nursing, or chronic hemolytic anemia (8).

6. Human Requirements

Body stores of folate in healthy persons have been reported as being between 5 to 10 mg, however body stores may be much higher. A considerable proportion of folate is stored in the liver. About 400 µg of folate a day is considered a suitable average intake (4). The normal human requirement is thought to be about 50 µg daily (5).

Folic acid (8) is usually adminsitered orally when oral adminsitration is not feasible or when malabsorption is suspected, the drug may be administered by deep 1M subcutaneous, or IV injection. A dilute solution of folic acid for oral or parental administration may be prepared by diluting 1ml of folic acid injection containing 5mg of folic acid per ml with 49ml of sterile water for injection to provide a solution containing 0.1mg of folic acid per ml.

Dosage of folic acid injection (sodiumfolate) is expressed in terms of folic acid in general, although patient response to folic acid therapy depends on the degree and nature of the deficiency. If proper corrective measures are undertaken folate deficient patients generally respond rapidly. During the first 24 hours of treatment, the patient experiences an improved sense of well-being, and within 48 hours, the bone marrow begins to become normoblastic. Reticolocytosis generally begins within 2-5 days following initiation of folic acid therapy.

To detect the presence of folate deficiency without concealing pernicious anemia in patients with megaloblastic anemia the usual oral or 1 M dosage of folic acid is 0.1-0.2 mg daily for 10 days. During this 10-day period the patient should maintain a diet low in folic acid and vit B_{12} content. Following adminsitration of these dosages of folic acid in patients with megaloblastic anemia, reticulocytosis reversion to normoblastic hematopoiesis the return of a normal hemoglobin indicates the presence of folic acid deficiency. The usual therapeutic dosage of folic acid for adults and children is 0.25-1mg daily.

In alcoholics, patients with hemolytic anemia or chronic infections, and patients receiving anticonvulsants, higher maintenance dosage may be required. Oral dosage of 3-15 mg daily have been recommended for patients with tropical sprue. In general, an oral dosage of 0.1 mg daily is considered sufficient as a nutritional supplement. To prevent megaloblastic anemia of pregnancy and fetal damage, up to 1mg of folic acid daily throughout pregnancy has been suggested.

7. Cautions

Folic acid is relatively nontoxic. Allergic reactions to folic acid preparations have been reported rarely and have included erythema rash, itching, general malaise, and bronchospatic respiratory difficulty. One patient experienced symptoms suggesting anaphylaxis following injection of the drug. Adverse GI and CNS effects have been reported rarely in the patients receiving 15 mg of folic acid daily for one month.

Folic acid should be administered with extreme caution to patients with undiagnosed anemia, since folic acid may obscure the diagnosis of pernicious anemia by alleviating hematologic manifestations of the disease, while allowing neurologic complications to progress. This may result in severe nervous system damage before the correct diagnosis is made. Adequate doses of vit B_{12} may prevent, halt or improve neurologic changes caused by pernicious anemia. (8).

Folic acid (4) should never be given alone or conjuntion with inadequate amounts of hydroxocobalamin for the treatment of megaloblastic or pernicious anaemia. Though folic acid produces a haemopoietic response, it fails to prevent the onset of subacute combine degeneration of the cord. Folic acid should not be given before a diagnosis has been fully established. The inclusion of folic acid in multivitamin preparations may be dangerous.

Large and continuous doses of folic acid may lower the blood concentration of vitamin B_{12}. Folic acid has been given to correct the folate deficiency associated with the use of anticonvulsants. Folic acid is usually well tolerated. (4).

8. Methods of Analysis

8.1 Elemental analysis

The elemental analysis of folic acid is as reported.

Element	Composition
C	51.70
H	4.34
N	22.22
O	21.75

8.2 Identification Methods

When folic acid is heated with formaldehyde in 2 N H_2SO_4 the yellow colour formed is relatively stable,

and absorbance at 397 nm Obeyed Beer's law in the 10-150 μg/ml range. (24).

8.3 Colourimetric Method

1. Folic acid (25) is extracted from tablets and injections with dil. alkali and reduced by zinc and HCl to 2,4,5, triamino-6-hydroxypyrimidine, which is then treated with ninhydrine to give a purple colour, and the extinction is measured at 555 nm. Beer's law is followed in the range 4.5 to 45 $\mu g/ml^{-1}$ of folic acid.

2. This method (26) is based on formation of a grayish-blue complex between folic acid in $NaoH-Na_2Co_3$ medium and folin-ciocalteu reagent, the absorbance is measured at 760nm and Beer's law is obeyed for 4.4 to 44 $\mu g\ ml^{-1}$ of folic acid. N-(4-Aminobenzoyl)glutamic acid (a decomposition product of folic acid) also form a colour with the reagent, if this compound is present folic acid must be seperated by TLC before ethe determination.

3. Folic acid (about 10 ug per ml) is determined (27) colorimetrically after oxidation with $KMnO_4$ diazotisation with $NaNO_2$, and coupling and colour development at pH 1.8 to 2.0. The colour is compared at 550 nm with that of a folic acid standard and a blank (3% K_2HPO_4 solution) as used for diluting sample and standard, treating similarly. The error for folic acid is 4.4%.

4. Hutchings et al (28) describes a chemical method of estimating folic acid. It was based on the observation that pterylglutamic acid and related compounds were cleaved by reduction with zinc and acid giving a pteridine and an arometic amine. The latter was estimated colorimetrically by reaction with N-naphthylendiamine.

Interference due to the presence of adenine, and nucleic acid was eliminated by reducing with titanous chloride instead of with zinc and acid. (29).

8.4 Spectophotometric Method

1. In this method the UV spectrum of folic acid solution in 0.1 N-NaoH exhibits characteristic extinction bands at 220, 256, 283 and 366 mu. The extinction at 256 and 283 mu are analytically the most useful as their ratio, which is nearly constant (1-02) for solution of the pure acid indicates the quantity of the sample. (30).

2. O. Hrdy (31) also describes the various spectrophotometric pharmacopoeial methods compared and statistically evaluated and their reliability is discussed.

3. The method is based on the reduction of folic acid with Zn dust and Hcl, the mixture is filtered, and the filterate is treated with (i) ethanolic dimethylamino cinnamaldehyde, (ii) $NaNo_2$, ammonium sulphamate and phloroglucinol, or (iii) NaoH, phenol and NaClO the absorbance of the product is measured at 520, 450 and 610 nm, respectively. (32).

4. Sastry et al describes the method which is intended for application to the assay of pharmaceutical preparations, involves mixing 15 ml of potassium hydrogen phthalate buffer solution (pH 3.1) 1 ml of aq. 0.1% catechol, 1 ml of 0.01N-iodine and 1.5 ml of the amino solution dilution of the mixture to 25 ml with H_2O, and after 5 to 30 minutes (depending on the amine), measured at 500-520nm (vs, reagent blank). (33).

8.5 Polarographic Method

1. Rozanski, L. et al (34) describes the method in which the finaly powdered sample, containg > 1 mg of folic acid was shaken for 30 minutes with 0.1M-HCl (25ml) aquous, 3% calcium lactate aquous, 8% Na_3PO_4 (1:1) buffer (225 ml) was added, and the mixture was heated at 50° for one or (for slow release tablets) two hours and filtered. A 20ml portion of the filterate was adjusted to pH 6.8 to 7.5 with aquous 10% ascorbic acid and after purging the solution with N_2, a polarogram was recorded from -0.4V (vs. the s.c.e); 5ml of a standared folic acid solution was then added and a second folic acid was calculated from

a given equation. The sensitivity of he method was about 0.1 ug ml^{-1} for a diffusion current of 1 nA, and accurate and reproducible results were obtained for 1.39 to 1.74 mg of folic acid.

2. Jozan et al (35) studied a method that a solution of the tablets in alkaline Na citrate solution (pH 12) was analyzed directly by polarography. Although insoluble additives interefered, their effect was constant for contents upto about 30%. The polarogram was recorded from -250 to -1050 mV (vs. the s.c.e) for folic acid. The half-wave potential (mV) was -483.

3. Folic acid in pharmaceutical preparations can also be determined polarographically. Dissolve one tablet (containing 30-60ug of folic acid, and usually Fe salts) in 10ml of 0.15 M- diethylenetriamine - N N N N'N"- penta acetic acid (disslve 60 g of the acid in NaOH, adjust the pH to 8 and dilute to 1:1) and dilute to 50 ml with 0.1M-acetate buffer of pH 5.5. De-aerate 20 ml portion with N_2, record on a.c. polarogram. (use of a phase-sensitive a.c. polarograph improves the sensitivity substantially), measure the peak height, and determine the folic acid content by use of a calibration graph (rectilinear over the range 20 nm to20 µm if a phase-sensitive a.c. polarograph is used) or by the standard, addition method. (36).

4. W.J. Mader et al (37) estimated folic acid polarographically except when iron is present with 1% tetramethylammonium hydroxide solution, pH 9.0 to9.5, as the base solution, it has a half-wave potential verses the S.C.E. of 0.98 volt with cadmium as an internal standard, the error of the estimation is 2%. (38).

8.6 <u>Fluorimetric</u> <u>Method</u>

1. U. Hla. Pe. etal describes the method for urinary folic acid. Urine is collected for 5 hours after administration (oral or intramuscular) of 5mg of folate, and an aliquot adjusted to pH 3.5 is applied to a column of activated Al_2O_3 equilibrated with 0.2 M-acetate buffer of pH-4. After washing the column with buffer solution, the folic acid is eluted with saturated $Na_2B_4O_7$ solution and the fluorescence is

developed by $KMnO_4$ oxidation (decolorising with H_2O_2) and measred at 470 nm (excitation at 365 nm). (38).

2. The fluorimetric determination of folic acid has been examined. On oxidation with $KMnO_4$ it is converted in to 2-amino-4-hydroxypteridine-6-carboxylic acid which flourescence strongly at 470 nm. When irradiated with light of wave-length 365 nm; the intensitiy of the fluorescene is proportional to the concentration. When interfering pigments are present, the oxidation product is isolated chromatographically. (39).

8.7 Radio-Assay

1. Serum folate was estimated by Dunn et al. (40). The serum was heated on a boiling water bath with lysin buffer solution of pH 10.5 to destroy the binding capacity of protein, then the mixture is diluted with [^{3}H] folate, B-lactoglobulin solution is added and after 45 minutes, the free and bound forms of folate are seperated by centrifugation with haemoglobin-coated charcoal; the supernatent solution is analysed for ^{3}H by a liquid scintillation technique.

2. In an other method a partially purified folate binding preparation from hog-kidney is incubated with serum and tritiated pterylglutamate in phosphate-ascorbate buffer, pH 7.6, at 4°C for 30 minutes in dark. Free tritiated pterylglutamateis removed by the addition of cold, albumin-coated charcoal suspension and centrifugation. Free and bound labelled pterylglutamate is measured by scintillation counting. (41).

3. In this method the assay of folic acid in plasma is based on inhibition by folic acid of the binding of ^{3}H-labelled folic acid to a folic acid specific protein obtained from milk. A commercial test kit is used and reproducible results were obtained in 600 assays on plasma from healthy men and women receiving tablets each containing 0-25 mg of folic acid. Results for normal subjects not receiving folic acid ranged from 3.2-20.2 ng ml^{-1} (average 6.8 ng ml^{-1}). This procedure is suitable for differenciating between anaemias due to deficiency of cyanocobalamin and of folic acid. (42).

4. Smultaneous radio-assay of serum vitamin B_{12} (cyanocobalomin) and folic acid was done by Gutcho et al. The procedure describes for determining both these vitamins in same 100 µl portion of serum. (43).

5. Hendel etal used the method in which antiserum was raised in rabbits immunised with a conjugate of folic acid and methylated bovin serum albumin. Erythrocytes and plasma samples were diluted with buffer solution and heated, the mixture were centrifuged and the supernatant solution were used in the assay; urine was assayed directly. The antiserum and sample were incubated for 2 hours at 4° with [^{3}H] folic acid. Poly ethylene glycol was added in the mixture, centrifuged, and the radioactivity of the precipitate was measured by liquid scintillation counting. (44).

6. Folic acid was also measured in human plasma and erythrocytes. Samples were homogenized with 0.05 M. phosphate buffer (pH 6.1) containing ascorbate. The homogenates were treated with chicken pancreas confugase and incubated for 2 hours at 37° and the enzyme action was stopped by heating the vessel in a boiling water bath. Folic acid was then determined by the radiometric and turbidmetric method. (45).

7. Waxman et al (46) also studied the serum folate levels and serum folic acid binding protein by ^{3}H-PGA (tritiated pterylglutamic acid) radio-assay.

8.8 Chromatographic Methods

8.8.1 Column Chromatography

Plasma folate compounds can be seperated from antibiotic and hypnotics by applying a sample solution in an agous NaCl to a column (10cm x 10mm) of TEAE-cellulose (cellex T. calbiochem) and carrying out gradient elution by allowing 0.5M-phosphate buffer of pH 6.5 to drip from an overhead container into a mixing chamber containing 500 ml of H_2O at 2 ml per minute while the elute leaves the column at the same rate. Folate is completely seperated from the other compounds, which appear in the first 40ml of elute, the folate present, apears later, in the 160 to 240ml fraction. (47).

8.8.2 Thin layer chromatography (TLC)

A summary of some of the TLC systems investigated for the analysis of folic acid are given in the table (4).

8.8.3 High Performance Liquid Chromatography (HPLC)

The different methods used to determine folic acid by HPLC are summarised in the table (5).

8.9 Thermal Analysis (DSC)

A differencial scanning calorimetry curve of folic acid was obtained Fig (8) on a Perkin-Elmer DSC-2c differencial calorimeter. Nitrogen was used as the purge gas. Scan was performed at the rate of 25°C/min. from 60-380°C. The DSC curve revealed an endothermic melting peak (Max. 193.38°C).

9. Acknowledgements

The authors appreciate the technical services extended by Mr. Babkir Awad Mustafa, College of Applied Medical Sciences, King Saud University, Riyadh. The authors are also very much thankful to Mr. Tanvir A. Butt, College of Pharmacy, King Saud University, Riyadh for typing the manuscript.

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Table (4): Summary of conditions used for the TLC of folic acid.

Plate	Developing solvent	Detection	Extd. solvent	R_f	Ref.
Cellulose powder	Butanol: acetic acid: water (4:1:5)	254 nm	-	0.1 at conc. > 1 µg per µl and 0.42 at conc. of 0.5 µg.	48
Silica gel G	Propanol: 10% aq. NH_3 glycerol (4:1:1) for 2 hours.	Green-yellow spot	10% $CuSO_4$ solution 2% aq. NH_3 (5:1).	0.41	49
Silica gel G 0.25 mm	Butanol: acetic acid: ethanol:water (250:1: 100:125).	550 nm	3% aq. Na_2HPO_4 (6 ml)	-	50
Kiesel gel G	Acetic acid: butanol: H_2O (1:4:5)	UV	-	-	51

Table (5) Summary of HPLC conditions for the determination of folic acid

Column	Mobile phase	Flow rate	Sample	Detection	Ref.
(30 cm X 4 mm) packed with μ Bonda pak C_{18} (10 μm)	5 mM tetrabutylammonium phosphate in aq. 30% methanol	1 ml/min	-	254 nm	52
μ Bondapak C_{18}	Acetonitile: 0.1 M Na. acetate buffer pH 5.7 (3:47)	-	Nutritional diet	365 nm	53
Stainless steel (30 cm X 4 mm id) packed with μ Bondapak C_{18}.	[($NaClO_4.H_2O$ 35.1 g)], [K_2HPO_4 (1.36 g)], [N.KOH (6.94 ml)] and [CH_3OH (40 ml)] diluted to 1 L with H_2.	-	-	254 nm	54

Continued /...

Continued (Table 5) ...

Analytical column (30 cm X 4 mm and guard column (5 cm X 4 mm) contained C_{18} bonded phase.	Amm. phosphate buffer solution	-	-	254 nm	55
(30 cm X 4 mm) of µ Bondapak C_{18}	[Tetrabutylamm. hydroxide (7.5 ml of aq. 40% solution)]-KH_2PO_4 (2.04 g)-[H_3PO_4 (4 ml of 3N)]-[methanol (240 ml)]-H_2O (to 1l) as mobile phase of pH 7.0.	1.5 ml/min	Multivitamin	280 nm	56
Silica gel C_{18}	Methanol: 4 mM Na heptane sulphonate in aq. 2% acetic acid (1:9).	-	Multivitamin	280 nm	57
Reversed phase column	0.05 M KH_2PO_4-0.25 M $NaClO_4$ buffer pH 7.2.	-	-	254 nm	58

Continued /...

Continued (Table 5)...

Ultraspher 1P and µ Bondapak phenyl	Phosphate buffer solution (pH 2.3) containing acetonitrile.	-	Selected food	Fluorescence	59
Pre-column (5 cm X 3 mm) of µ Bondapak phenyl/ Corasil (30 µm).	0.042 M-$NaClO_4$, 1.5 mM-KH_2PO_4 and 1.6% of methanol adjusted to pH 7 with 1M-KOH	-	-	280 nm	60

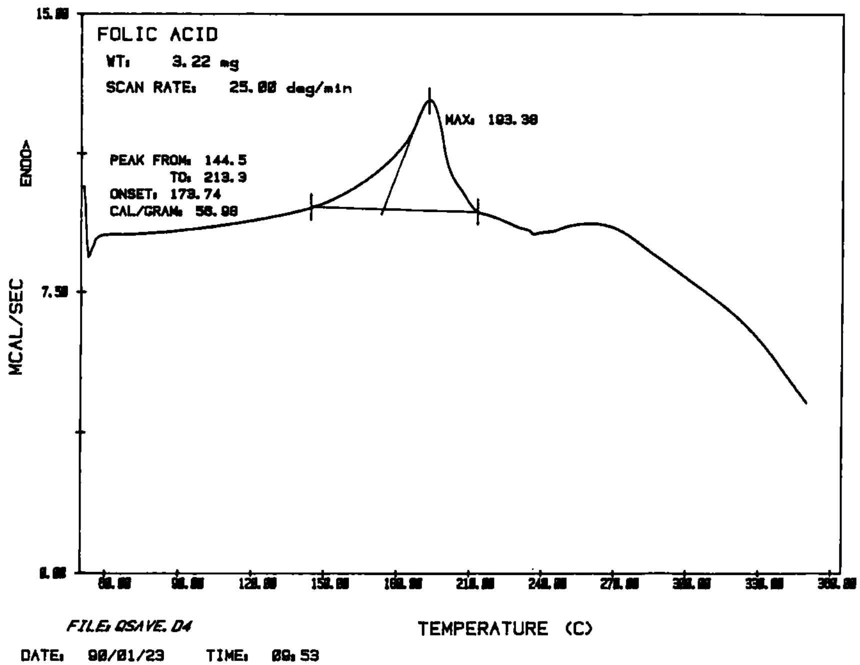

Fig. 8: Differencial Scanning Calorimetry Curve of Folic Acid

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ANALYTICAL PROFILE OF LOBELINE HYDROCHLORIDE

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LOBELINE HYDROCHLORIDE

CONTENTS

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

2-[6-(2-Hydroxy-2-phenylethyl)-1-methyl-2-piperidinyl]-1-phenylethanone.
2-[6-(β-hydroxyphenethyl)-1-methyl-2-piperidyl] acetophenone.
2-(2-Hydroxy-2-phenylethyl)-N-methyl-6-phenacyl piperidine.
Ethanone 2-[6-(2-hydroxy-2-phenylethyl)-1-methyl-2-piperidinyl]-1-phenyl-, [2R-[2α,6α (S*)]].
Names of the acids forming the salts are added after the chemical names e.g.
2-[6-(β-hydroxyphenethyl)-1-methyl-2-piperidyl] acetophenone hydrochloride.

1.1.2 Generic Names

Lobeline hydrochloride; Alpha lobeline hydrochloride; α-Lobeline hydrochloride; Inflatine hydrochloride.

1.1.3 Trade Names

Lobron; Zoolobelin (for the hydrochloride)
Bantron; Glenden; Habit-X; Lobatox; Lobeton; Lobidan; Toban o-t-c; unilobin (for the sulfate).

1.2 Formulae

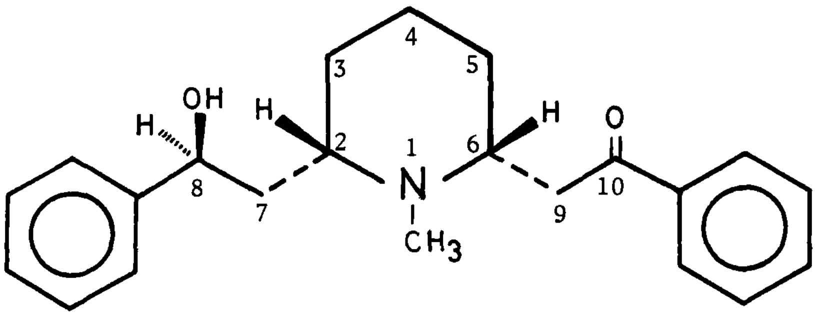
1.2.1 Empirical

$C_{22}H_{27}NO_2$	(Lobeline)
$C_{22}H_{28}Cl\ NO_2$	(Lobeline hydrochloride)
$C_{44}H_{56}N_2O_8S$	(Lobeline sulfate)

1.2.2 Structural

Lobeline is one of the pyridine-piperidine alkaloids. Its chemical structure was proposed by Wieland and co-workers (1-3) and has been confirmed by the total synthesis of the

alkaloid, which was first carried out by the same group (2-5) and later by others (6-10).



1.2.3 CAS Registry Number

[90-69-7] Lobeline
[134-63-4] Lobeline hydrochloride
[134-64-5] Lobeline sulfate

1.2.4 Wiswesser Line Notation (11)

T6NTJ A B1YQR & F1VR
*LV (Lobeline).
T6NTJ A B1YQR & F1VR *LV
& GH (Lobeline hydrochloride).

1.2.5 Absolute Configuration

The absolute configuration of natural (-)-lobeline and of (±)-lobeline were deduced from chemical degradation, synthesis and co-relation (12,13).
The configuration of natural (-)-lobeline was established as (2*S*, 6*R*, 8*S*)-8,10-diphenyl-lobelionol (12).
The configuration of racemic lobeline was determined as (2*S*, 6*R*, 8*S*)-8,10-diphenyl-lobelionol and its enantiomer (2*R*, 6*S*, 8*R*)-8,10-diphenyl-lobelionol (13).
The absolute configuration of (-)-lobeline is shown above.

1.3 Molecular Weight

337.47 (Lobeline)
373.92 (Lobeline hydrochloride)
773.0 (Lobeline sulfate)

1.4 Elemental Composition

Lobeline : C, 78.30%; H, 8.07%; N, 4.15%; O, 9.48%.
Lobeline hydrochloride : C, 70.67%; H, 7.55%; Cl, 9.48%; N, 3.75%; O, 8.56%.
Lobeline sulfate : C, 68.37%; H, 7.30%; S, 4.15%; N, 3.62%; O, 16.56%.

1.5 Appearance, Color and Odor

- Lobeline occurs as needles(from alcohol, ether, benzene (14), it has a bitter taste.
- Lobeline hydrochloride occurs as rosettes of slender needles (from alcohol (14) or as a white odorless crystalline or granular powder with a bitter taste (15).
- Lobeline sulfate occurs as hygroscopic crystals (from alcohol (14) or as white crystalline powder, odorless with a bitter taste.

2. Physical Properties

2.1 Melting Range

- Lobeline : 130-131° (1,2,14)
- Lobeline hydrochloride, the following data have been reported:-
  178-180° (14); not lower than 180° (15);
  182° (1,2,11,16); not below 178° (17).

2.2 Eutectic Temperature

The eutectic temperature of lobeline hydrochloride is recorded as follows (17):

	Microscope Hot Stage	Hot Bar
Benz.	135°	-
Sal.	146°	162°
Dic.	-	134°

Benz. = Benzanilide; Sal. = Acetaminosalol;
Dic. = Dicyandiamide.

2.3 Solubility Data

- Very slightly soluble in water; soluble in hot alcohol; in chloroform; in ether and in benzene (Lobeline (1,2,14).
- One gram of lobeline hydrochloride dissolves in 40 ml water; 12 ml alcohol; very soluble in chloroform and very slightly soluble in ether (14,15).
- One part of lobeline sulfate is soluble in about 30 parts of water; slightly soluble in alcohol (14).

2.4 pH Range

A 1% solution of lobeline hydrochloride in water has a pH of 4.0 to 6.0 (17).

2.5 Specific Optical Activity

The following data have been reported:

$[\alpha]_D^{15} = -42.85°$ to $-43°$ (EtOH) for lobeline (1,2,11,14).

$$\left.\begin{array}{l} [\alpha]_D^{20} = -43° \text{ (alcohol, C=2) (14)} \\ [\alpha]_D = -56 \text{ to } -58° \text{ (1\% solution) (17)} \end{array}\right\} \text{for lobeline HCl}$$

$[\alpha]_D^{20} = -25°$ (C=2) for lobeline sulfate (14).

2.6 Crystal Structure

The crystal structure of lobeline hydrochloride as $C_{22}H_{27}NO_2 \cdot HCl \cdot H_2O$ is reported (18). It was recrystallized from water as transparent needles.
Lobeline hydrochloride exhibited the following data:

Space group	a(A°)	b(A°)	c(A°)	Density $(g.cm^{-3})$ observed	Density $(g.cm^{-3})$ calculated
$P\,2_12_12_1$	8.1	14.2	18.0	1.24	1.25

No. of formula units/unit cell = 4.

The above data were obtained from oscillation and Weissenberg photographs, while the density was determined by floatation. Calculation of the unit cell contents from the cell dimensions and the observed density requires a molecular of water to be associated with each formula unit (18).

2.7 X-Ray Powder Diffraction

The X-ray diffraction pattern of lobeline hydrochloride was determined with a Philips X-ray diffraction spectrogoniometer equipped with PW 1730 generator. Radiation was provided by a copper target (Cu anode 2000 W, γ=1.5480 A°). High intensity X-ray tube operated at 40 KV and 35 MV was used. The monochromator was a curved single crystal (PW 1752). Divergance slit and the receiving slit were 0 and 0.1° respectively. The scanning speed of the goniometer used was 0.02 2θ per second.
The X-ray pattern of lobeline hydrochloride is presented in Fig. 1. Interplanner distance and relative intensity are tabulated in table 1.

Table 1 : X-Ray Powder Diffraction Pattern of Lobeline Hydrochloride

d(A°)	I/Io	d(A°)	I/Io
16.54	100.0%	3.40	23.9%
12.01	45.4%	3.35	26.5%
6.67	57.4%	3.30	26.9%
6.51	14.4%	3.24	27.5%
5.99	23.6%	2.99	22.9%
5.39	54.9%	2.75	18.2%
5.25	40.4%	2.39	18.2%
5.06	49.5%	2.32	12.1%
4.61	45.9%	2.03	12.8%
4.40	52.1%	2.00	11.4%
4.23	49.9%	1.99	10.3%
4.02	60.5%	1.82	9.5%
3.71	57.8%	1.61	6.5%
3.65	22.8%	1.49	5.3%
3.52	22.9%	1.43	5.8%

d = interplanner distance, I/Io = relative intensity (based on the highest intensity of 100).

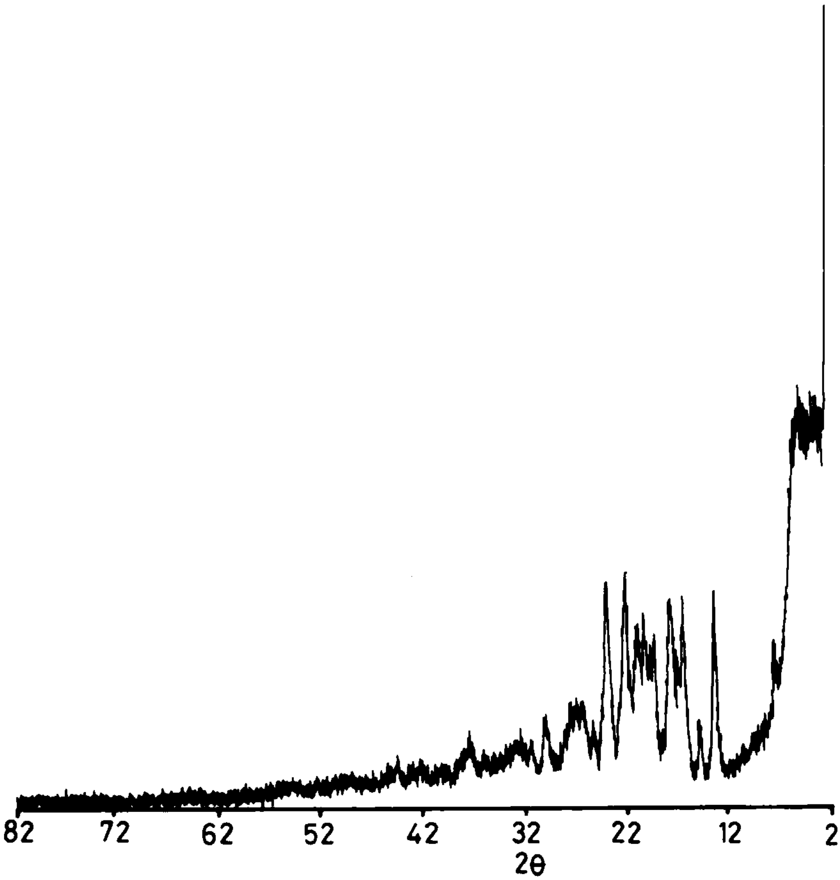


Fig. 1 : X-Ray Diffraction Pattern of Lobeline HCl.

2.8 Spectral Properties

2.8.1 Ultraviolet Spectrum

The UV absorbance spectrum of lobeline hydrochloride in methanol was scanned from 200 to 400 nm using a Pye-Unicum SP 8-100 Spectrophotometer (Fig. 2).
Lobeline hydrochloride exhibited the following absorptivity values (Table 2).

Table 2 : UV absorptivity values

max nm	log ε	A(1%, 1 cm)
245	4.08	311
280	3.05	30.3

Other reported UV data for lobeline:-

Solvent	max nm	Ref.
Methanol	245(13183); 280(1413)	(11)
0.1NHCl	249(A_1^1 = 416)	(19)
0.1NH_2SO_4	249(A_1^1 = 421)	

2.8.2 Infrared Spectrum

The IR spectrum of lobeline hydrochloride as a KBr-pellet (1 : 200 mg) was recorded on a Perkin Elmer 580B Infrared Spectrophotometer (Fig. 3).
Assignment of the functional groups have been correlated with the following frequences (Table 3).

Table 3 : IR Characteristics of Lobeline

Frequency Cm^{-1}	Functional group
3340	OH (stretch)
3065-2925	CH (stretch)
2818	H_3C-NH^+
1680	C=O

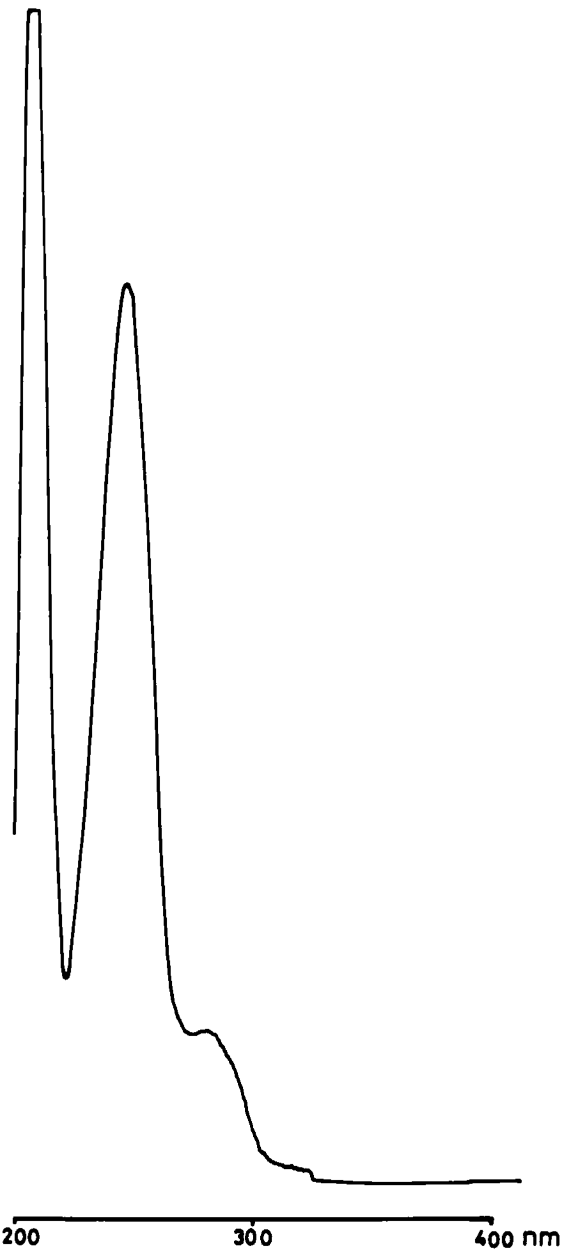


Fig. 2 : UV Spectrum of Lobeline HCl (In Methanol).

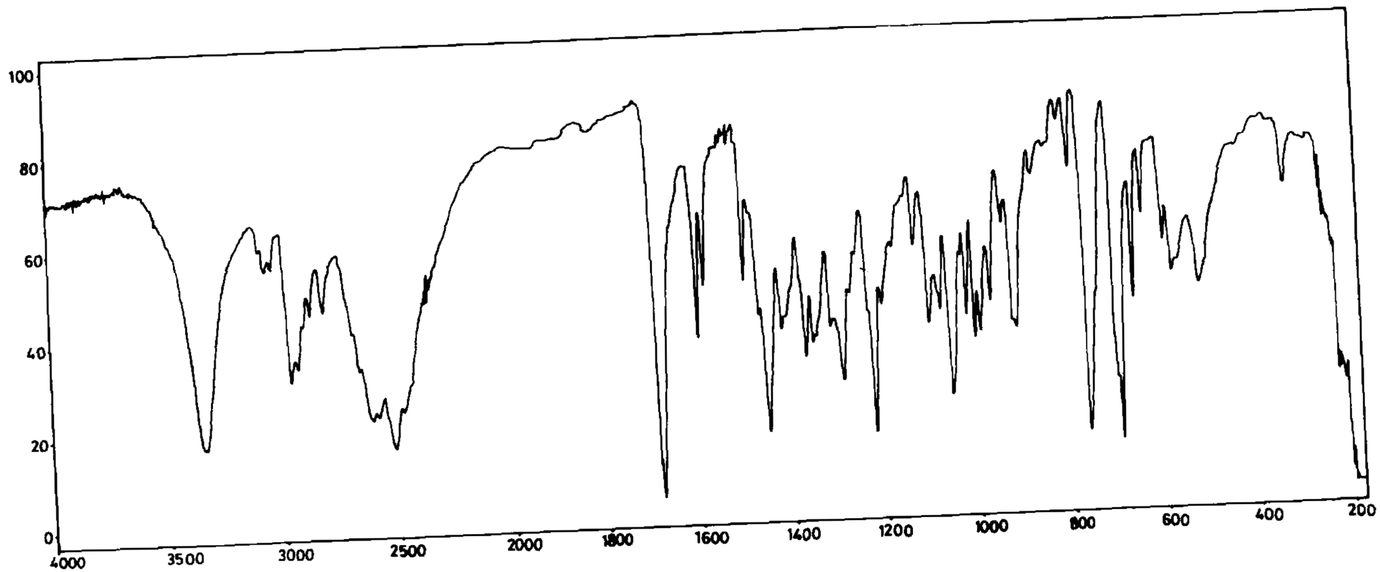


Fig. 3 : IR Spectrum of Lobeline HCl (KBr-Pellet).

Frequency Cm^{-1}	Functional group
1600,1585	C=C (aromatics)
1500,1452	C H (bending)

The IR of lobeline hydrochloride exhibited the following other characteristic absorption bands:- 2580, 2510, 1420, 1370, 1350, 1290, 1220, 1205, 1135, 1105, 1080, 1055, 1020, 1005, 995, 970, 945, 920, 875, 820, 795, 765, 695, 670, 645, 600, 582, 525 Cm^{-1}.
Clarke (19) reported the following principal peaks for lobeline base in KBr disc:- 1687; 1211; 1115 and 700 Cm^{-1}.

2.8.3 Nuclear Magnetic Resonance Spectra

2.8.3.1 ^{1}H-NMR Spectra

The proton spectra of lobeline hydrochloride were recorded, once in $CDCl_3$ on a Varian FT80A (80 MHz) NMR spectrophotometer (Fig. 4), and another in DMSO-d6 on a Varian XL-200 (200 MHz) NMR spectrophotometer (Fig. 5), using TMS as an internal reference with both. The proton chemical shifts are assigned and presented in table 4.

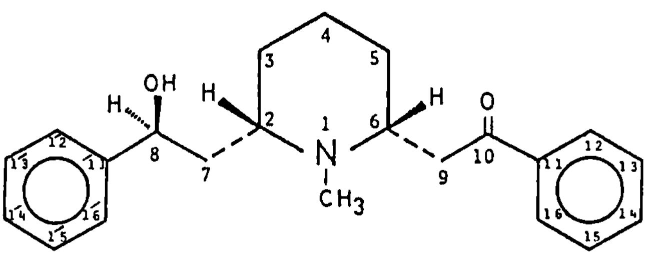


Table 4 : ^{1}H-NMR Characteristics of Lobeline.

Proton assignment	Chemical shifts δ (ppm) 80 MHz	200 MHz
2 aromatic H at C_{12} & C_{16}	8.03-8.09(d)	8.04-8.10(d)
8 aromatic H at $C_{13,14,15}$ $C_{12',13',14',15',16'}$	7.27-7.55(m)	7.24-7.72(m)

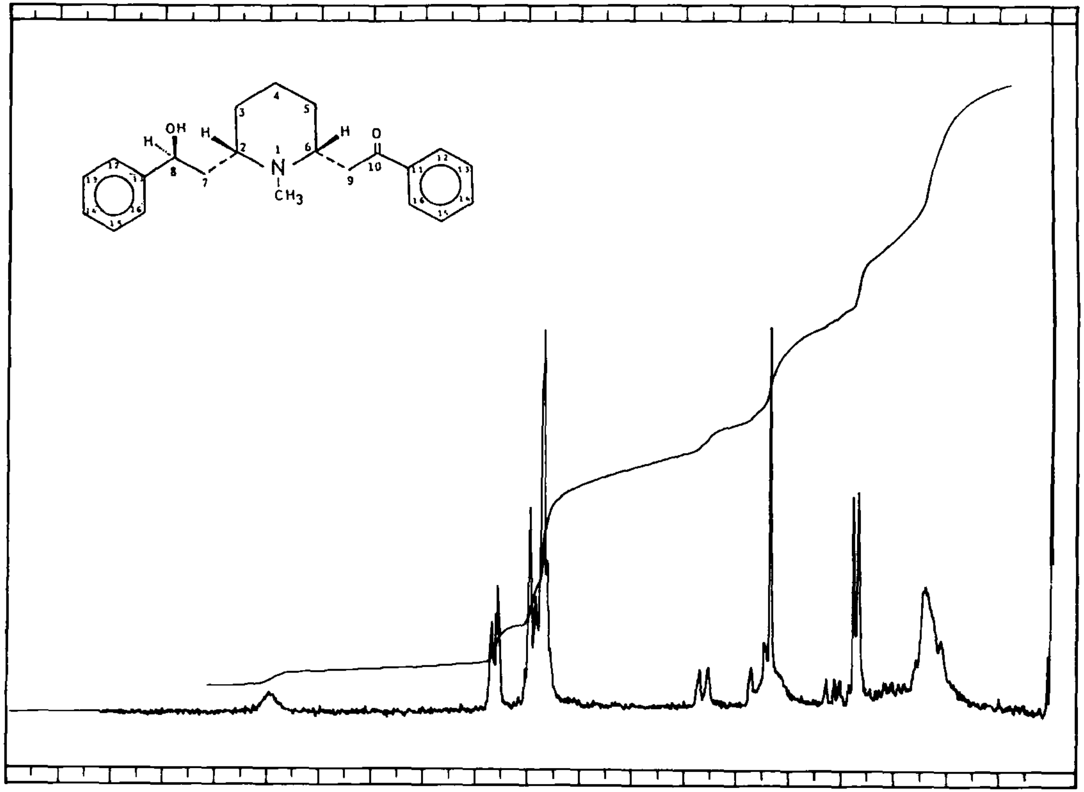


Fig. 4 : ^{1}H-NMR Spectrum (80 MHz) of Lobeline.

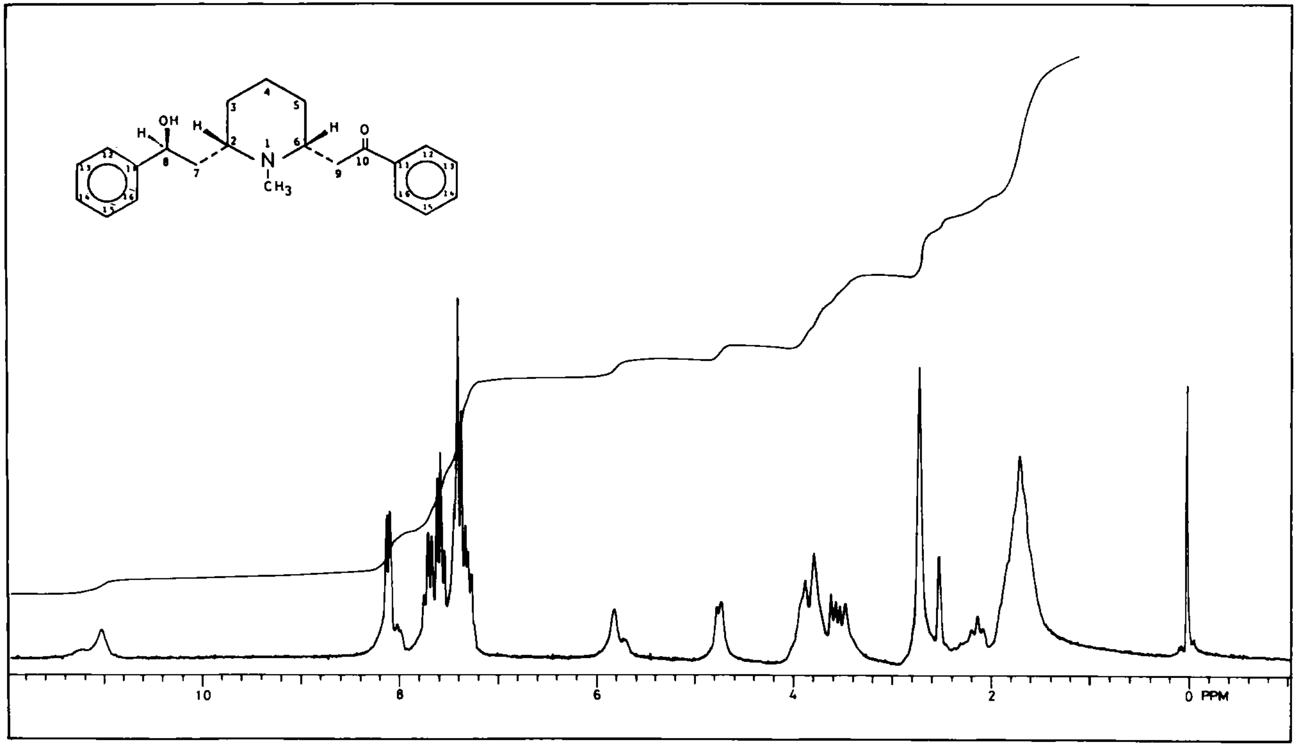


Fig. 5 : ^{1}H-NMR Spectrum (200 MHz) of Lobeline.

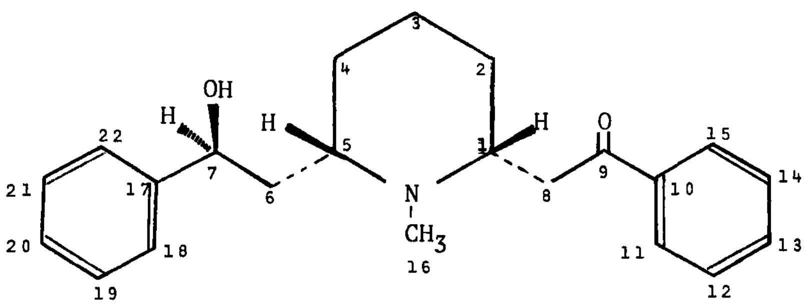
Proton assignment	Chemical shifts δ (ppm) 80 MH_z	200 MH_z
H at C_2 & H at C_6	-	5.8(d)
H at C_8	4.07(d)	4.73(d)
2H (methylene) at C_4 & C_9	-	3.82-3.86(d)
2H (CH_2) at C_7	-	3.56(m)
$N\text{-}CH_3$	2.81(s)	2.70(s)
2H (CH_2) at C_3 & C_5	CH_2 envelope 1.70-1.89	CH_2 envelope 1.7-1.89

s = singlet, d = doublet, m = multiplet

^1H-NMR spectrum of lobeline hydrochloride can be seen in one reference (20).

2.8.3.2 Carbon-13 NMR Spectrum

The ^{13}C-NMR spectrum of lobeline hydrochloride in DMSO-d_6 was recorded on a Varian XL-200 NMR spectrophotometer, using TMS as an internal reference. The spectrum is shown in Fig. 6. The carbon chemical shifts were assigned and presented in table 5.



Carbon-13 NMR data for lobeline is not reported elsewhere.

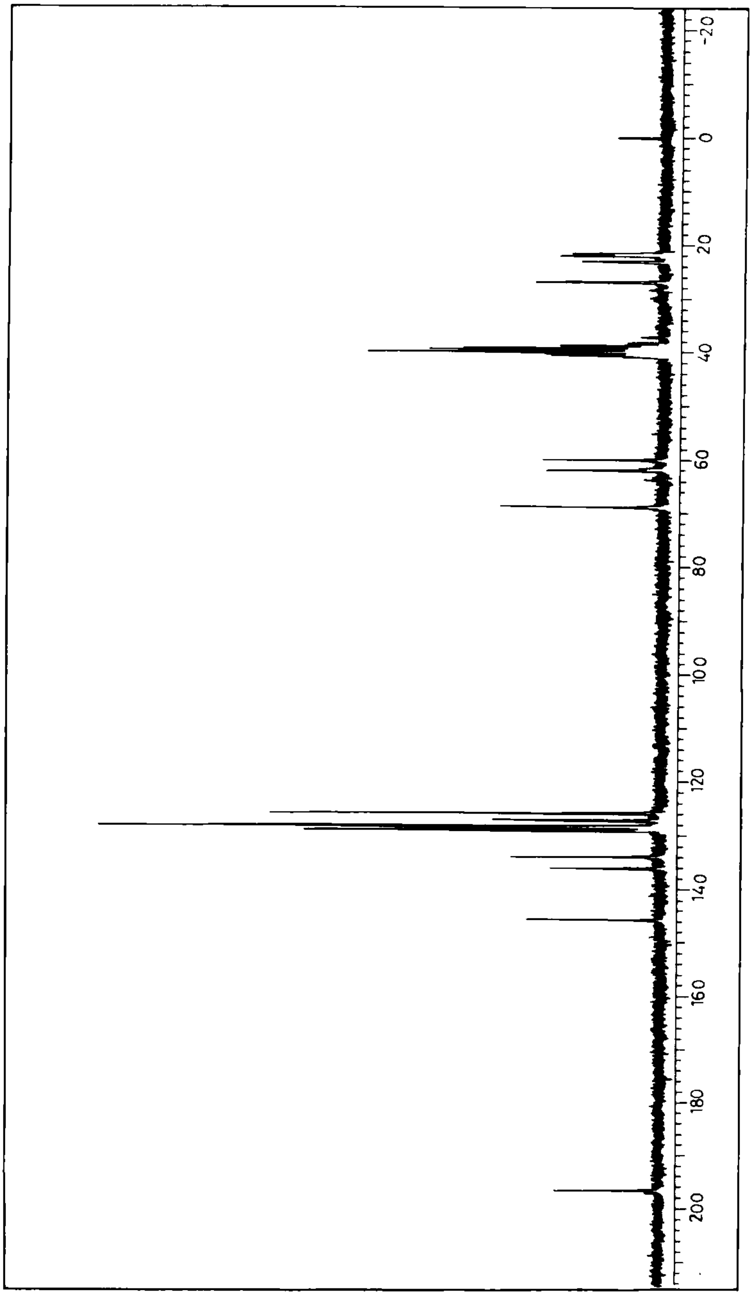


Fig. 6 : Carbon-13 NMR Spectrum of Lobeline.

Table 5. Carbon-13 Chemical Shifts of Lobeline

Carbon Assignment	Chemical Shift δ (ppm)	Multiplicity
C_9	196.18	Singlet
C_{10}	145.39	Singlet
C_{17}	135.84	Singlet
C_{13}	133.68	Doublet
C_{11},C_{15}	128.67	Doublet
C_{12},C_{14}	128.06	Doublet
C_{20}	127.94	Doublet
C_{18},C_{22}	126.82	Doublet
C_{19},C_{21}	125.51	Doublet
C_7	68.67	Doublet
C_1	61.71	Doublet
C_5	59.77	Doublet
C_8	40.56	Triplet
C_6	39.90	Triplet
C_{16}	27.00	Quartet
C_2,C_4	22.05	Triplet
C_3	21.76	Triplet

2.8.4 Mass Spectrum

The electron-impact ionization (EI) mass spectrum of lobeline hydrochloride is presented in Fig. 7.
The spectrum was obtained using a Finnigan MAT 5100 series GC/MS spectrometer operating with an ionization potential of 70 eV.
The spectrum shows a base peak at a mass/charge (m/z) ratio of 96.2.
The molecular ion peak of lobeline (337) is absent or undetectable, this could be explained either due to the alcoholic nature of the substance (21) or the molecule underwent fast McLafferty rearrangement.
The most prominent ions and their relative intensities are listed in table 6. Some proposed ion fragments of lobeline are shown in Fig. 8.

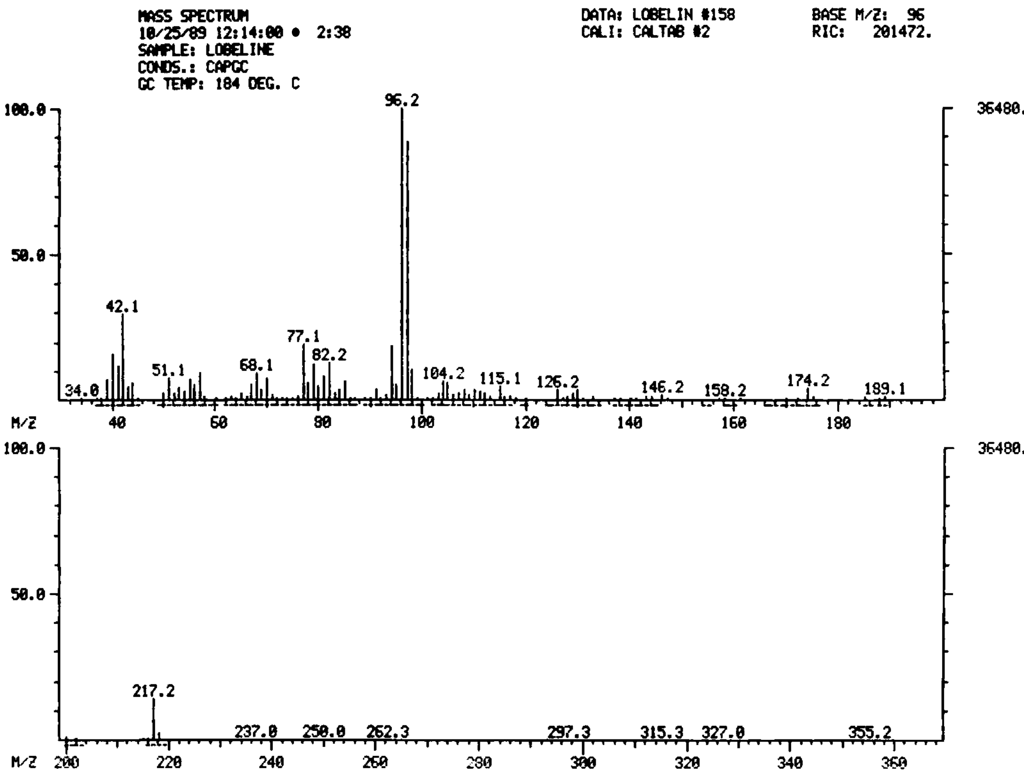


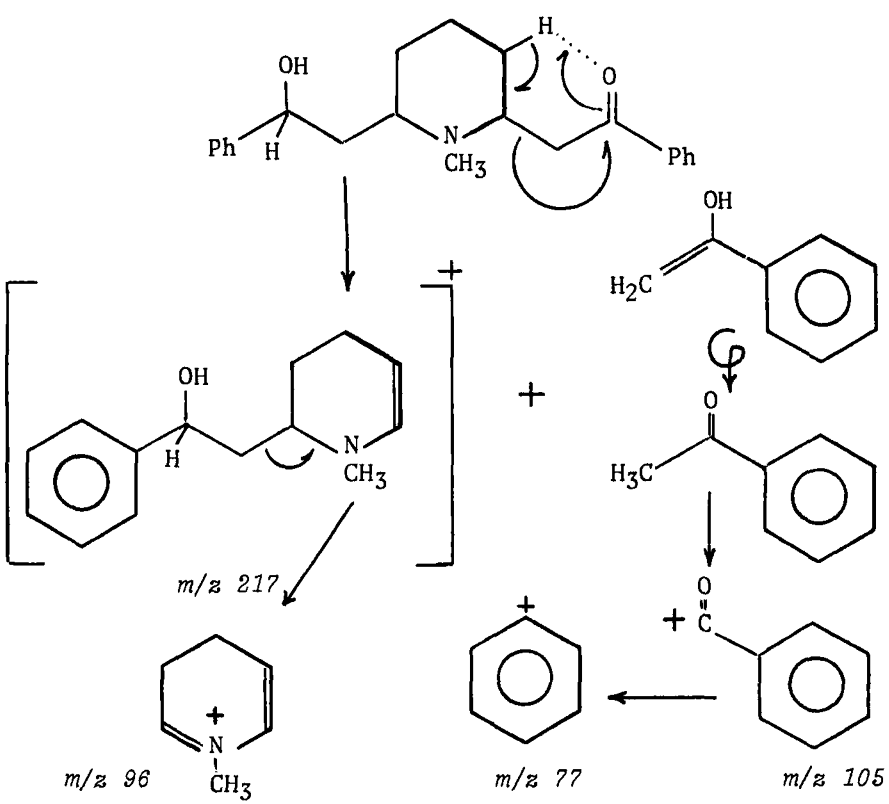
Fig. 7 : Mass Spectrum of Lobeline.

Table 6. The Mass Fragments of Lobeline

m/e	Relative % Intensity	m/e	Relative % Intensity
42	30.1	82	13.7
51	6.9	94	19.2
58	9.6	96	100.0
68	9.6	97	87.7
70	6.9	98	11.0
77	19.2	104	6.3
79	12.3	105	5.7
81	8.2	217	14.0

Principal peaks of mass spectrum of lobeline at m/z 96, 105, 77, 97, 216, 42, 218, 51 were reported (22).

Fig. 8 Lobeline ion fragments



3. Isolation of Lobeline

Lobeline occurs in lobelia which is also known as Indian tobacco. It is the herb of *Lobelia inflata L.* family Campanulaceae. Lobelia contains about 0.24-0.4% alkaloids, the most important of which is lobeline (23).
Lobelia is still official in several Pharmacopoeias including the BP 1980 (24).

- The powdered lobelia herb is moistened with water slightly acidified with acetic acid and left for sometime. The resulting mass is then pressed and the process of moistening and pressing is repeated. The collected pressed liquids (extracts) are filtered.
- The filtrate is rendered alkaline with sodium bicarbonate and extracted with ether. The ether extract is then shaken with water acidified with sulfuric acid. The acid layer is rendered again alkaline with sodium bicarbonate and exhaustively shaken with ether (a step of purification).
- The ether is evaporated to dryness, and the resulting yellow oily residue is dissolved in water acidified with hydrochloric acid, filtered and then shaken with chloroform *(chloroform will extract only lobeline hydrochloride leaving salts of the other alkaloids in the aqueous layer).*
- The chloroform extract is evaporated to dryness under vacuum, and the resulting brownish oily residue is stirred repeatedly with twice its volume of hot water at 60°. The aqueous solution is kept for sometime in a vacuum desicator over sulfuric acid, when lobeline hydrochloride crystallizes out.
- If lobeline base is required, lobeline hydrochloride is dissolved in warm water, rendered alkaline with sodium hydroxide solution and extracted with ether. This is evaporated to dryness and the residue is recrystallized from alcohol or benzene (1,5,25).

The isolation scheme of lobeline is presented in Fig. 9.

Lobeline can be purified on a chromatographic column of neutral alumina and elution is carried out with benzene (26).

Other methods for the isolation of lobelia alkaloids including lobeline have been described in many patents (16,27).

The mutarotation of optically active lobeline has been studied (28,29). Both *(-)-cis and (+)-trans-8,10-diphenyllobelionol* undergo mutarotation. The rate of mutarotation of *(-)-cis-8,10-diphenyllobelionol* is increased in hydrophyllic solvents and in the presence of hydroxyl ions.

Fig. 9 The Isolation Scheme of Lobeline HCl

Powdered lobelia + H_2O
+ Acetic acid, *Leave and press*

Marc

Extract +
sod. bicarbonate
shake with ether

Ether layer
shake with H_2O + H_2SO_4

residue

Ether layer

Aq. acid layer +
sod. bicarbonate, *shake*
with ether

Ether layer
Evaporate to dryness,
dissolve in H_2O + HCl,
Filter

acid layer

Precipitate

Filterate
shake with $CHCl_3$

Chloroform layer
Evaporate
Brown residue of
lobeline HCl
Digest with H_2O at 60°, *leave* in a dessicator,
crystals of lobeline hydrochloride.

Aq. acid layer
(other alkaloids)

4. Structure of Lobeline

The following chemical reactions were carried out mostly by Wieland and co-workers (2-5,9 *) to deduce the structures of lobeline, lobelanine and lobelanidine.*

Lobeline [I] can be reduced with sodium amalgam to produce the diol lobelanidine [II]. Lobeline [I] can also be subjected to mild oxidation with chromic acid in acetic acid to give the diketone lobelanine [III]. *Both lobelanidine [II] and lobelanine [III] accompany lobeline [I] in the plant (*1,30,31).

Lobelanidine [II] and lobelanine [III] can be converted into lobeline [I] by oxidation and reduction respectively.

Degradation of lobeline [I] (by removing the phenacyl side chain), gives (2S,8S)-8-phenyl-lobelol [IV] (12).

Lobelanine [III] undergoes Hofmann elimination to produce the nitrogen-free derivative, 1,7-dibenzoylhept-1,6-diene [V] which is upon mild catalytic hydrogenation yielded the dione 1,7-dibenzoylheptane [VI] (3,16,31).

Lobelanine [III] yields the dioxime (Bis oxime) [VII] which undergoes a Beckmann rearrangement and hydrolysis of the diamide so formed yields N-methylpyridine dicarboxylic acid [VIII] and aniline [IX] (3,30).

Lobeline [I] or lobelanine [III] when heated to 140° with molten benzoic acid or at 125° with excess dilute hydrochloric acid, gives rise to acetophenone [X] (2,16).

Lobelanine [III] on oxidation with potassium permanganate produces 2 moles of benzoic acid (16).

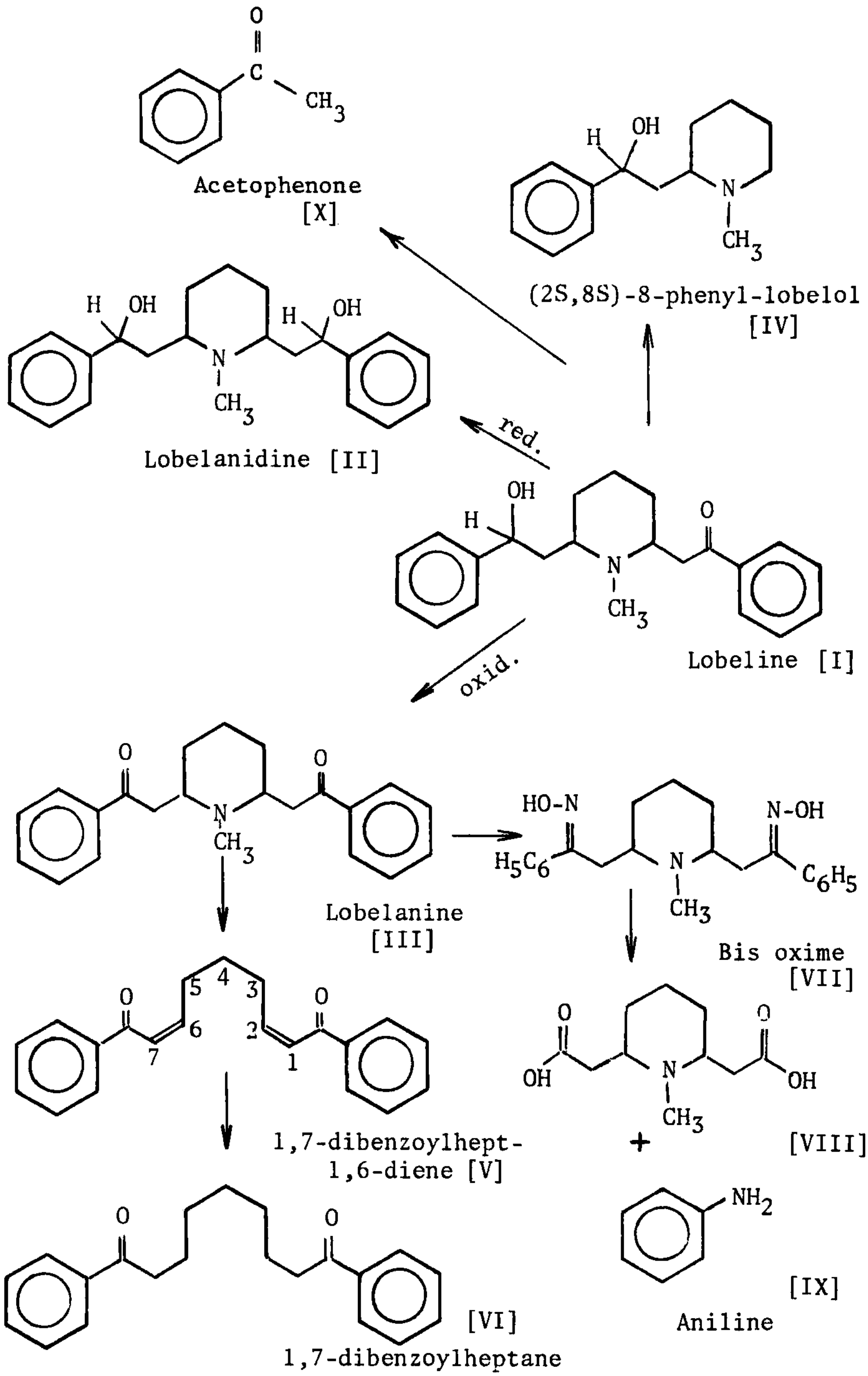
All above reactions are shown in Fig. 10.

5. Synthesis of Lobeline

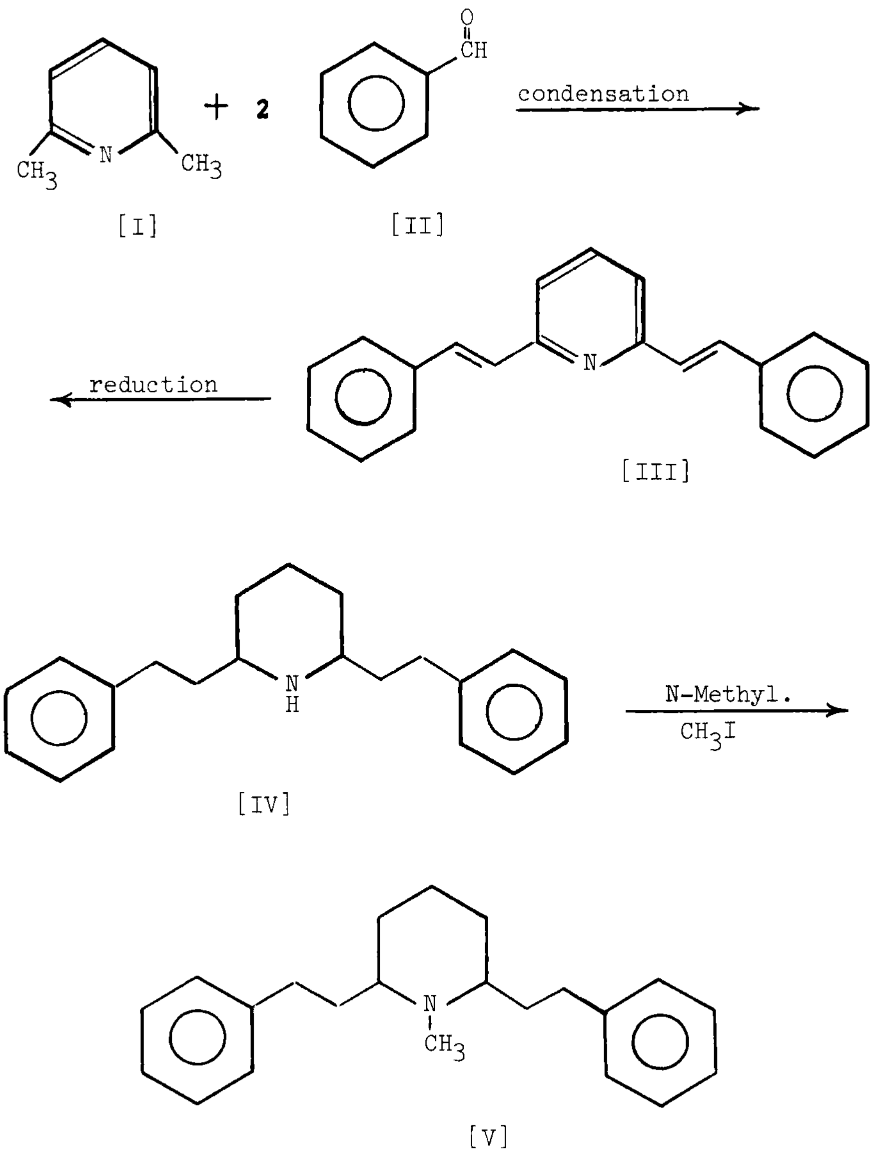
Several different syntheses of lobeline have been achieved (2-10). The most efficient procedure which is used on an industrial scale was reported in 1935 by Schopf and Lehman (8 *). The first total synthesis of lobeline via lobelanidine was achieved in 1929 by Wieland and Drishaus (* 4 *). These authors have first synthesized the parent compound lobelan as follows:-*

2,6-Dimethylpyridine (lutidine) [I] was condensed with two molecules of benzaldehyde [II] to afford 2,6-distyryl-pyridine [III]. This was hydrogenated with sodium in alcohol to give a mixture of *cis*-and *trans*-2,6-diphenethyl-piperidine (norlobelan) [IV]. The cis-form upon treatment with methyl iodide was converted into lobelan [V]. Lobelan [V] has also been obtained when 2,6-diphenethyl-1-methylpyridinium *p*-toluene sulfonate [VI] is hydrogenated over Adam's catalyst (6).

Fig. 10. Chemical Reactions of Lobeline



Scheme I : Total Synthesis of Lobelan



Synthesis of lobelan is shown on scheme I.

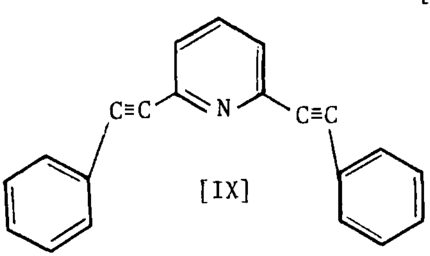
The first total synthesis of lobeline was carried out via lobelanidine (4):-

Ethyl glutarate [I] was condensed with acetophenone [II] in the presence of sodamide to give the adduct 1,7-dibenzoylhepta-2,6-dione [III]. This upon treatment with ammonia at 100° gave the piperidine derivative [IV]. Catalytic hydrogenation of the latter over platinic oxide produced the glycol [V] which was isolated in two isomeric forms : α-norlobelanidiene and β-norlobelanidiene. Reduction of the β-form in ether solution with aluminum amalgam gave rise to norlobelanidine [VI] (identical in every respect with the naturally occurring base) (5). N-methylation of [VI] produced lobelanidine [VII], which was then transformed into lobeline [VIII] by mild oxidation with chromic acid.

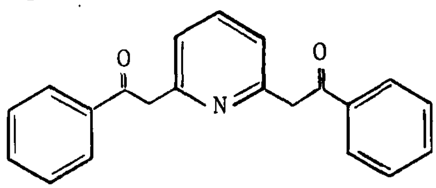
This first synthesis is presented in scheme II.

The above synthesis has been considerably simplified as follows (7):-

2,6-Distyrylpyridine added bromine to give 2,6-distyryl-pyridine tetrabromide which was shaken with alcoholic potash to afford 2,6-di-β-phenethinylpyridine [IX]. Hydration with sulfuric acid (by heating for 10 minutes) yielded 2,6-diphenacylpyridine [X]. Catalytic reduction of [X] over a platinic oxide-barium sulfate catalyst gave 2,6-di [β-hydroxy-β-phenethyl] pyridine which was further reduced to norlobelanidine [VI].



[IX]

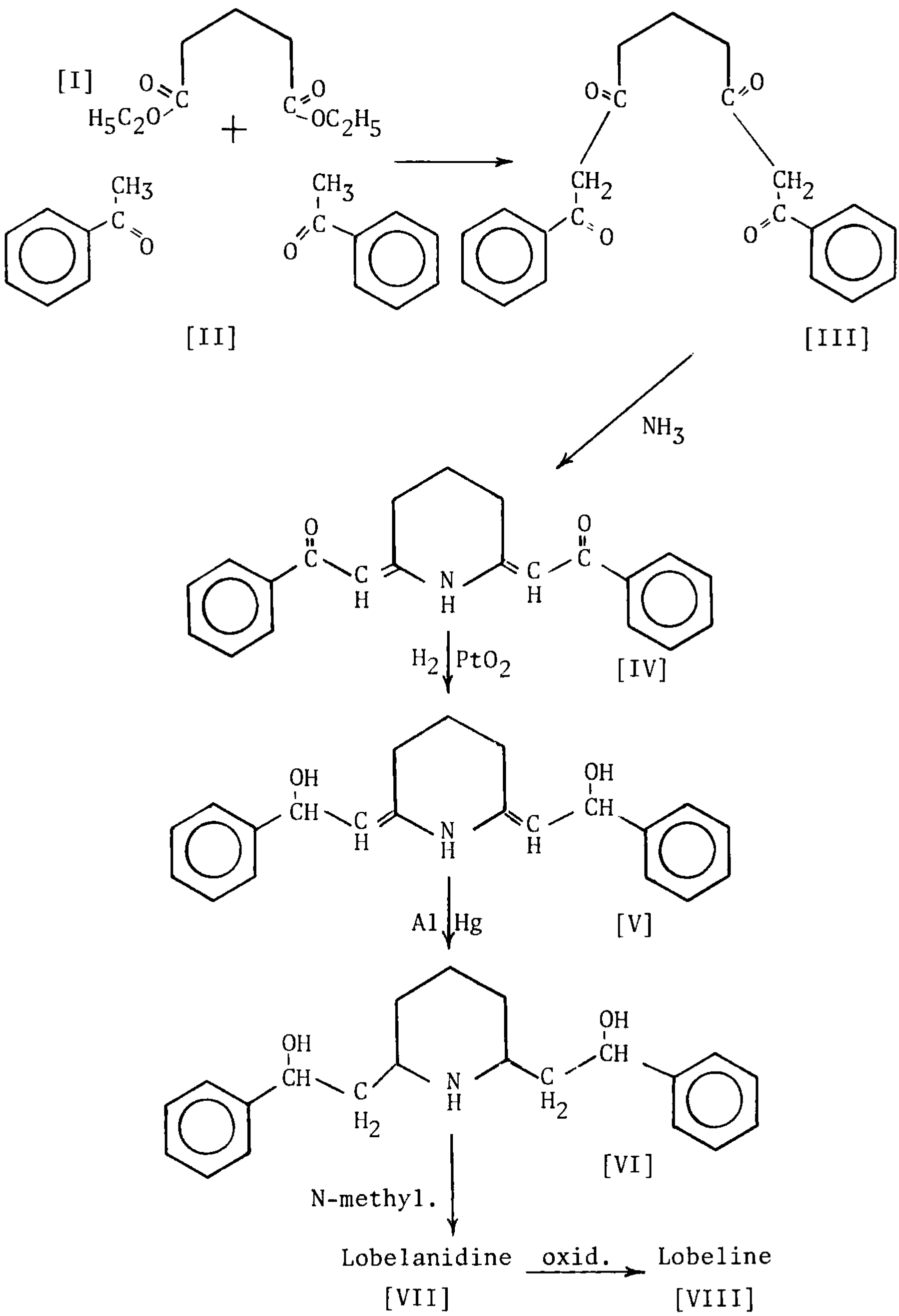


[X]

Commercial synthesis of lobeline has been achieved by an elegant method which involved a Mannich condensation and Robinson-type biomimetic reaction (8):

A mixture of glutaric dialdehyde [I], benzoylacetic acid [II] and methylamine hydrochloride [III] was kept in a buffer solution at pH 4.0 and 25° for 8 hours, gave

Scheme II : Total Synthesis of Lobeline via lobelanidine



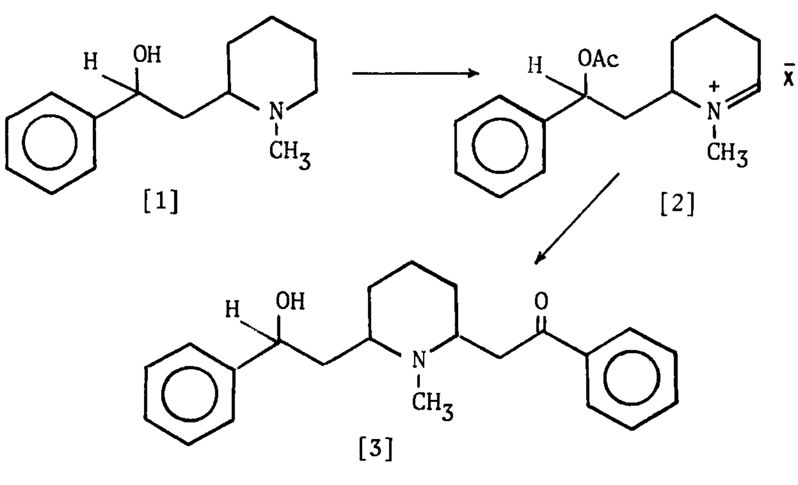
lobelanine [IV] in one step, in 90% yield. Reduction of [IV] with sodium amalgam afforded lobeline [V].

This commercial synthesis is presented in scheme III.

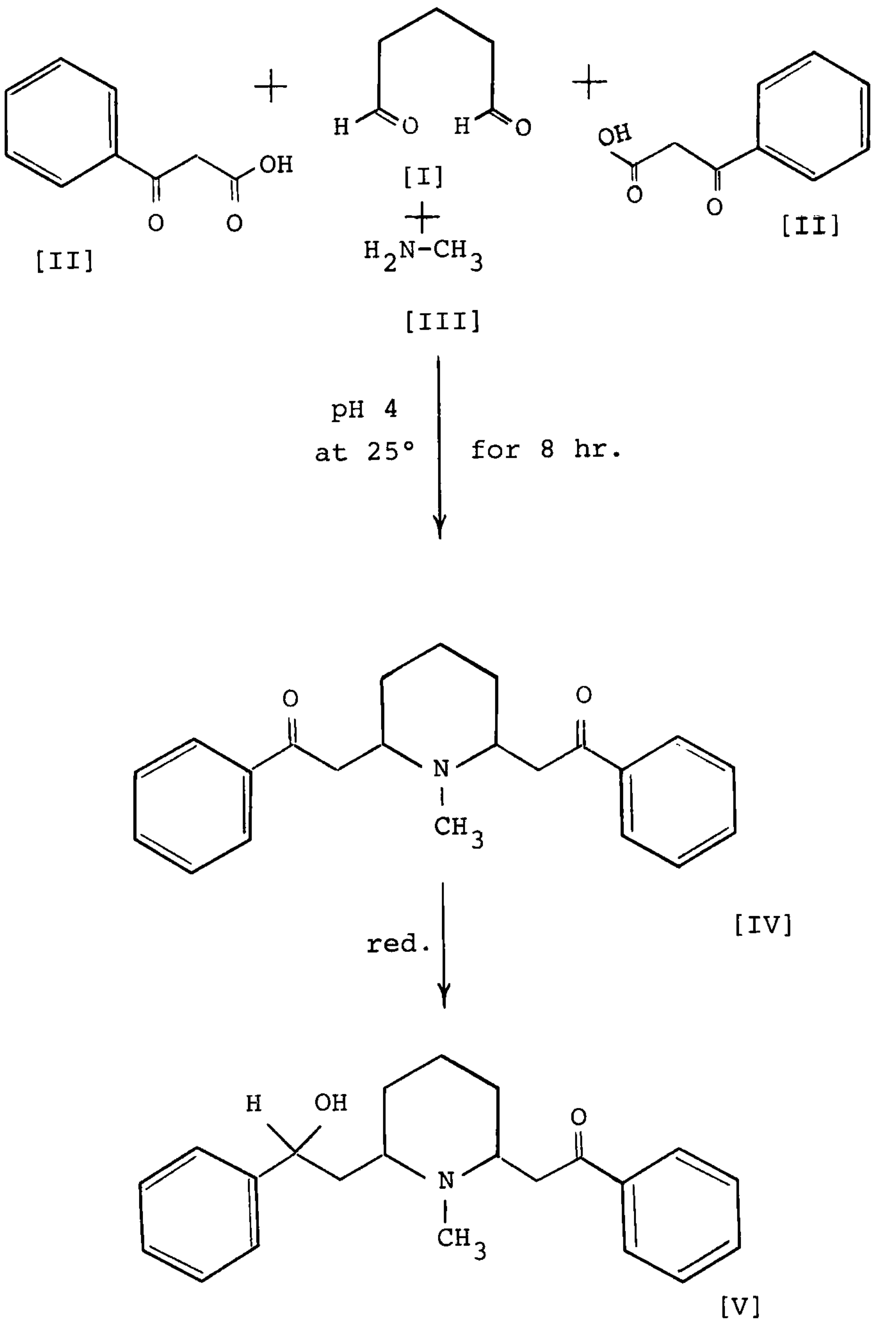
A recent synthesis of lobelanine has been carried out (10). Lobelanine can then be partially reduced to afford lobeline (1).
Hepta-1,6-diyne [1] was treated with 2 moles of ethylmagnesium bromide (Grignard reaction) [2] to produce the *bis*-Grignard derivative [3]. This upon condensation with 2 moles of redistilled benzaldehyde [4] gave 1,9-diphenylnona-2,7- diyne-1,9-diol [5]. The diol [5] was oxidized with a solution chromium trioxide (containing concentrated H_2SO_4) to furnish 1,9-diphenylnona-2,7-diyne-1,9-dione [6]. This acetylenic diketone was partially hydrogenated in benzene with Lindlar's catalyst to give *cis, cis*-1,9-diphenylnona-2,7-diene-1,9-dione [7]. The diketone [7] was treated with methylamine to produce lobelanine [8]. Reduction of [8] with sodium amalgam afforded lobeline [9].

This recent synthesis is presented in scheme IV.

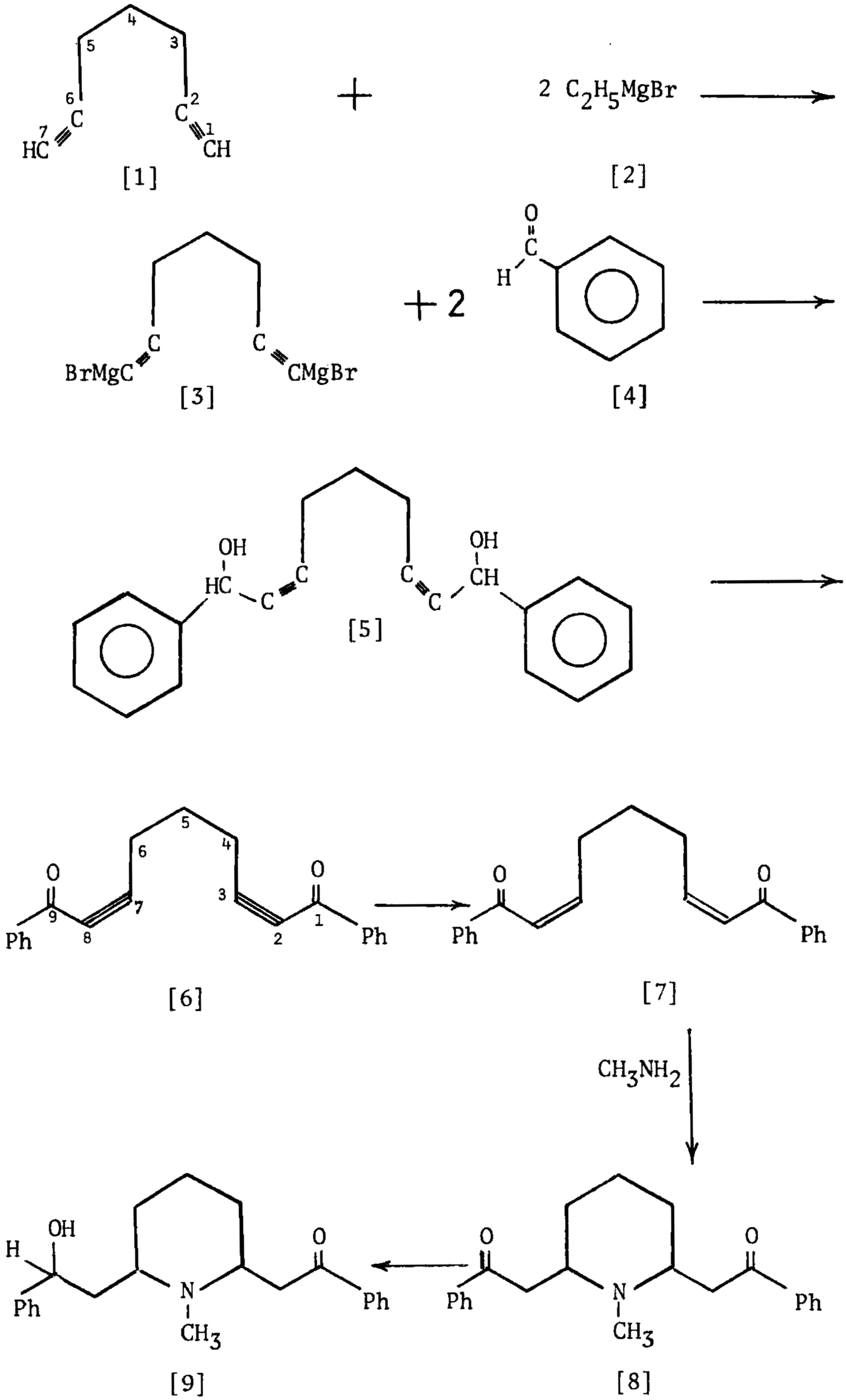
A partial synthesis of (-)-lobeline from (±)-sedamine has been described (12). (±)-Sedamine [1] was acetylated to yield the (±)-O-acetylderivative which was dehydrogenated by treatment with mercuric acetate in acetic acid to the 1,6-dehydroderivative [2]. Condensation of [2] with benzoyl acetic acid gave (±)-O-acetyl lobeline. Saponification of the latter, followed by chromatographic separation and mutarotation at room temperature afforded (-)-lobeline [3]. This was recrystallized from water-free ether to furnish pure (-)-alkaloid.



Scheme III : Commercial Synthesis of Lobeline.



Scheme IV: Recent Synthesis of Lobeline



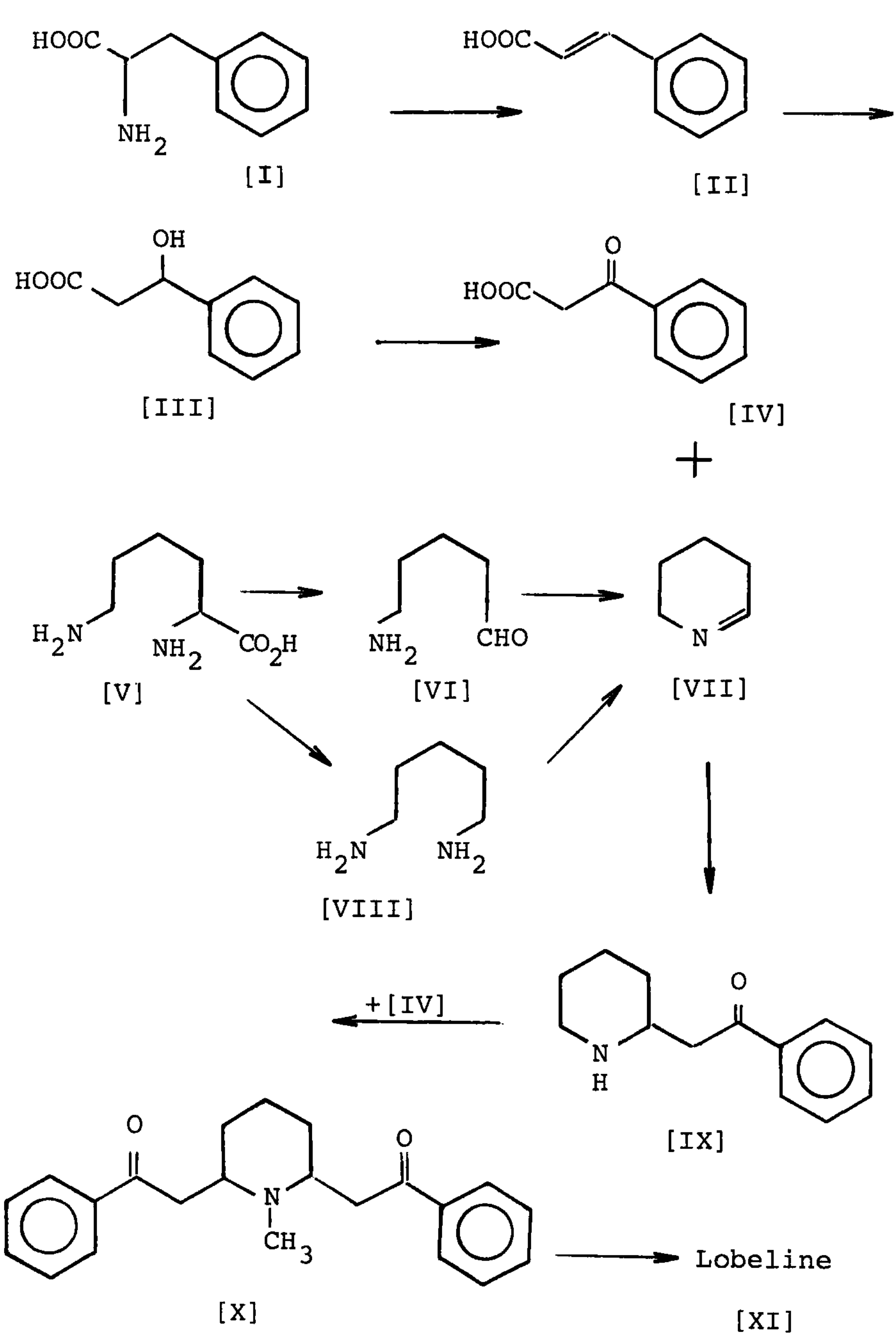
6. Biosynthesis of Lobeline

Robinson was the first to suggest that lobeline in the plant *Lobelia inflata* is arised from lysine and phenylalanine (32).
Leete later proposed that lobeline is built up in nature from benzoic acid and acetate or from acetate alone (33). Tracer studies have demonstrated that lobeline is derived from phenylalanine and lysine (26,34), thus confirming Robinson's suggestion. The studies have specifically shown that the piperidine nucleus of lobeline is derived from lysine and the two C_6-C_2 units are derived from phenylalanine (26). Further radioactive studies have established the biosynthetic pathway of lobeline in *Lobelia inflata* (35). *This pathway is presented in scheme* V .
Phenylalanine [I] on deamination with the enzyme phenylalanine ammonia-lyase *(which has been isolated from plant sources)* (36) yields transcinnamic acid [II]. Hydroxylation of [II] gives 3-hydroxy-3-phenylpropionic acid [III]. *(This has previously been isolated from Lobelia)* (37). The latter upon biological oxidation affords benzoylacetic acid [IV].
Lysine [V] is converted either via 5-aminopentanal [VI] or via pentane-1,5-diamine [VIII] into 2,3,4,5-tetrahydropyridine [VII].
Condensation of [IV] with [VII] yields the amino-ketone [IX] which on condensation with another mulecule of benzoylacetic acid [IV] affords norlobelanine, which upon N-methylation yields lobelanine [X]. This is transformed by partial reduction into lobeline [XI].

Incorporation of a number of the above intermediates into lobeline in lobelia plants has been demonstrated (35). Upon feeding in separate experiments each of (±)-[3-^{14}C] phenylalanine [I]; [3-^{14}C] cinnamic acid [II]; 3-hydroxy-3-phenyl [3-^{14}C] propionic acid [III]; [2-^{14}C] lysine [V] and 2,3,4,5-tetrahydro [2-^{14}C]-pyridine [VII] to separate *lobelia inflata* plants, radioactive lobeline was isolated from each experiment.

The incorporation of labelled lobelanine [X] into lobeline [XI] in high yield has also been reported (38).

<u>Scheme V</u> : Biosynthesis of Lobeline



7. Pharmacology

This section has been re-written by Dr. Zaki (39).

Lobeline mainly stimulates the respiratory center by direct central stimulation of the center in the medulla oblongata and by reflexly from the chemoreceptors in the carotid (40,41) and aortic bodies (42,43). This at therapeutic doses of lobeline leads to increase in both the rate and depth of respiration (44). Larger dose may induce cough.

Lobeline causes direct central stimulation of the medullary vagal center, vaso-motor center and chemoreceptor trigger zone (CRTZ) of the vomiting center which produces nausea and vomiting. Mild general central stimulation leads also to tremors (39,45).

Lobeline is a weak peripheral ganglionic stimulant. It produces different pharmacological effects depending on the dose and the state of the functional activity of the organs supplied by the ganglia. These include both sympathetic and parasympathetic effects due to stimulation of the autonomic ganglia by depolarization only and when large doses are used (46,47).

a) Sympathetic effects: are mainly on the cardiovascular system through sympathetic ganglionic stimulation, increased catecholamine release *(norepinephrine, epinephrine and dopamine)* from the adrenal medulla and stimulation of norepinephrine transmitter release. This leads to increase cardiac output and blood pressure (39,43).
b) Parasympathetic effects: are mainly manifested on the gastrointestinal tract and sweat glands in form of increased salivation and motility of the intestine with diarrhea. Its diaphoretic action is due to stimulation of the sweat gland secretion (48,49).

It has been reported that lobeline administered to human subjects caused a slight but definite suppression of appetite (anorexigenic action) (50). This suppression of appetite can arise from a number of mechanisms known to be elicited by lobeline (51):-

- Action on the central nervous system, probably on the appetite center or on CRTZ of the emetic center.
- Action on the adrenal medulla, causing hyperglycemia.
- Action on gastrointestinal tract, directly affecting nicotinic receptors.
- Action on pulmonary receptors, causing reflex inhibition of intestinal motility (48).

The most recent application of lobeline *(as the hydrochloride or sulfate salt taken orally)* is to administer it as a smoking deterrent (to control symptoms in patients undergoing withdrawal treatment for the tobacco habit) (50,51,52).

Large toxic doses of lobeline leads to both ganglionic and neuro-muscular blockade by excessive depolarization with severe hypotension, shock, coma and paresis or paralysis (24,39).

Lobelia is the dried aerial parts of *Lobelia inflata* L., it contains not less than *0.25%* of total alkaloids, calculated as lobeline (53).
Lobelia is still official in several pharmacopoeias, these include Austrian, Belgian, British, Brazilian, Egyptian, French, Polish, Portugese and Spanish pharmacopoeias (24).

It is used mainly as *Ethereal lobelia tincture* which contains *0.05 to 0.075%* of alkaloids calculated as lobeline (24).

Lobelia is incorporated in some cough mixtures as sedative expectorant. Its therapeutic value is due to reflex stimulation of the bronchial secretion modifying its physical character to be easily expelled. It has also a central stimulation effect on the cough center that forcibly helps to clear the air passages avoiding chest complications (39).

Storage

Lobeline hydrochloride should be kept in a tightly-closed containers protected from light (17).

Lobeline hydrochloride injections should be kept preferably in single-dose hermetically-closed containers or in multiple-dose containers protected from light (17).

Solutions for injections are sterilized by filteration and supplied in single dose alkali-free containers, protected from light, no bacteriocide should be added (15).

8. Methods of Analysis

8.1 Identification

The following identification tests have been mentioned under lobeline hydrochloride (17).

- Warm about 1 ml of a 1.0% solution with a few drops of sodium hydroxide TS; the aromatic odor of acetophenone is perceptible.
- Dissolve a few mg in 1 ml of sulfuric acid and add 1 drop of formaldehyde; a red color is produced (sensitivity 0.1 μg) (19).
- To 1 ml of 1.0% solution add a drop of ammonia TS; a milky liquid is obtained which crystalizes on standing for some time. Melting-temperature of the crystals, after washing with a little water and drying, about 120°.
- Yields the reactions characteristic of chlorides.

The following tests are used for the identification of lobeline and salts:-

- When drops of Froehde's reagent are added to few crystals of lobeline; a rose red color is produced which changes to a blue color (25).
- When drops of Erdmann's reagent are added to a few crystals of lobeline; a faint green color is produced which intensifies on warming.
- Ammonium molybdate reagent gives with lobeline a grey color which changes to purple (sensitivity 1.0 μg) (19).
- Ammonium vanadate solution gives with lobeline a grey color (sensitivity 0.5 μg) (19).

8.2 Microcrystal Formation

A 0.4% w/v solution of lobeline HCl was prepared for the microcrystal tests. 1 to 2 drops of this solution was treated with equal drops of the specific reagent on a microscopal glass slide, after about 5 minutes, the crystals so formed were microscopically examined (54).

- Potassium cyanide reagent (5%) furnished clusters and some irrigular rectangles (plate A).
- Sodium carbonate solution (5%) produced clusters (plate B), Clarke (19) reported rosettes with this reagent.
- Wagner's reagent gave clusters and prisms (plate C).
- Disodium hydrogen phosphate furnished rosettes (plate D).

Microcrystals of Lobeline

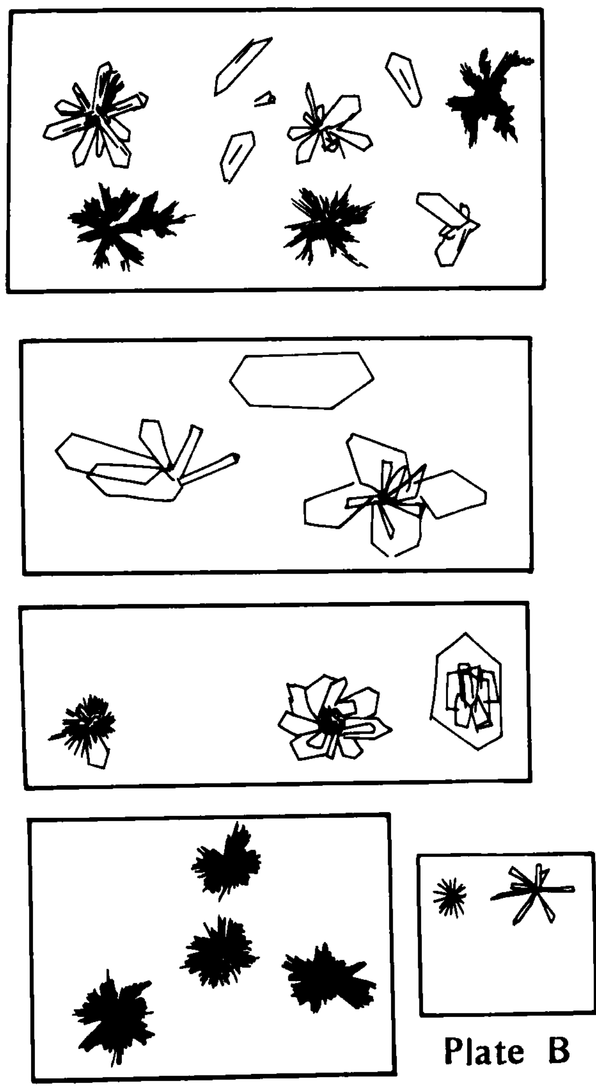


Plate A X40

Plate C

Plate D

Plate B

8.3 Titrimetric Determinations

8.3.1 Aqueous Titration

Markwell in 1936 (55) developed an aqueous titration method to determine the total alkaloids of lobelia. The method was modified and the use of 0.02N sulfuric acid as titrant was recommended (56).

The B.P. (53) has adopted this modified method for the assay of lobelia as follows:

The total lobelia alkaloids (from 10g fine powder) are extracted first with a mixture of four volumes of ether and one volume of ethanol (96%) and then after purification with chloroform. The chloroform layer is removed by distillation and the resulting residue of total alkaloids is dissolved in ethanol (2 ml), 0.01M sulfuric acid (10 ml) is added and the excess of acid is titrated with 0.02M sodium hydroxide, using methyl red solution as an indicator.

Each 1 ml of 0.01M sulfuric acid is equivalent to 0.006749 g of lobeline $C_{22}H_{27}NO_2$.

Lobelia alkaloids can also be estimated by acid-base titration (57,58).

The extracted total alkaloids are titrated with 0.1N HCl using methyl red as an indicator.

Each 1 ml of the acid = 0.03372 g of alkaloids calculated as lobeline.

Lobeline hydrochloride can be assayed after purification on a chromatographic column (59) as follows:

Lobeline hydrochloride (0.1 g) is dissolved in 95% aqueous acetone (10 ml) and applied to a column of basic alumina (Woelm 5 g). Elution is carried out with the same solvent. The eluate is diluted with water (25 ml) and titrated with 0.1 N HCl using methylred-methylene blue (6:1) as an indicator.

8.3.2 Non-aqueous Titration

Lobeline hydrochloride is assayed by the non-aqueous titration method (17):

An accurately weighed quantity of the salt is dissolved in glacial acetic acid. Acetous mercuric acetate (10 ml) and dioxan (20 ml)

are added. Titration to the end point is carried out with 0.1N acetous perchloric acid and after adding 2-3 drops of acetous crystal violet.

Each ml of 0.1N acetous perchloric acid is equivalent to 0.03739 g of lobeline hydrochloride, $C_{22}H_{27}NO_2$, *HCl.*

Good results of estimation in the non-aqueous media are also obtained by treating the total alkaloids with dimethylaminobenzene and titrating with 0.05N *p*-toluene sulfonic acid (60).

Lobeline hydrochloride as injections can be titrated in non-aqueous media either with 0.005 N 4-(benzenesulfonyloxy) benzene sulfonic acid or with 0.01 N 3,4-dichorobenzene sulfonic acid using dimethyl yellow in both titrations as indicator (61).

It has been claimed that these acids gave better results than titration with toluene-p-sulfonic acid (61).

The use of other derivatives of sulfonic acid as titrants for lobeline and other alkaloids has also been recommended (62). These derivatives include : 6-nitrotoluene-m-sulfonic acid; 4-chloro-2,5-xylenesulfonic acid and 3,6-dichloro-2,5-xylene sulfonic acid.

Other non-aqueous titration have also been reported (63).

8.3.3 Conductimetric Titrations

Lobeline hydrochloride in injections and in tablets can be determined either by direct conductimetric titration with 0.01 N or 0.005 N NaOH in ethanol medium or by addition of 0.01 N NaOH and ethanol, followed by conductimetric titration with 0.01 N HCl. The method can be also applied for the determinations of other hydrochlorides of several alkaloids (64).

8.4 Gravimetric Determinations

An assay method based on precipitation of lobelia alkaloids with silicotungstic acid has been developed by Mascre' in 1930 (65). In this method, the total alkaloids of lobelia are precipitated with 5% silicotungstic acid (in the cold). The resulting precipi-

tate is filtered off after 12 hours and incinerated. The weight of the ash multiplied by 0.414 gives the content of alkaloids calculated as lobeline.

The above method was further modified to determine the alkaloidal content of galenic preparations of *Lobelia inflata* after extraction of the alkaloids (66).

Barra (67) used the same method to determine lobeline, but he has simplified the extraction procedure prior to precipitation.

A combined precipimetric-titrimetric method was reported to determine the lobelia alkaloids in drugs (68):-
Precipitation of lobelia alkaloids with silicotungstic acid was first effected. The silicotungstates so formed were collected, decomposed with ammonia and extracted with ether. The residue after evaporation of ether was dissolved in dilute sulfuric acid and determined titrimetrically.

Complex thiocyanate salts as precipitating reagents for determining lobeline have been reported (69). The use of tripotassium hexathiocyanate for determining several alkaloids is recommended (69).

Other gravimetric determinations were also reported (70,71).

8.5 Polarographic Methods

Several alkaloids and salts including lobeline are determined qualitatively and quantitatively by a polarographic method (72).
Concentrations of 0.001 to 1% of alkaloids are separated quantitatively on aluminum electrode as anode (wrapped in parchament) and a steel one as cathode. The alkaloids collected at the cathode are washed, dried and their melting points determined for identification.

An oscillopolarographic study of some alkaloids including lobeline has been reported (73).
A dropping mercury electrode served as a polarizable electrode and a graphite one served as a reference. All alkaloids gave characteristic cuts in alkaline solutions (0.8 M KOH; 0.5 M LiOH; 5.5 M NaOH).

8.6 Spectrophotometric Methods

8.6.1 Colorimetric Determinations

A colorimetric method for the determination of lobeline has been reported. This method depends on degradation of lobeline into acetophenone and measuring the latter (74). Acetophenone derived from lobeline is mixed with the reagent 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl (5 ml). After standing for 2 hours, the mixture is shaken with carbon tetrachloride (10 ml).
Upon separation, the extract is washed with water, dried with anhydrous sodium sulfate and the clear supernatant liquid is measured against a blank at 370 nm.

A combined paper chromatographic-colorimetric determination of lobeline has been recommended (75) and this is as follows:

Preparation of chromatograms
Paper sheets are impregnated with a mixture solution containing formamide (9 ml), ammonium formate (0.8 g), formic acid (1 ml) and absolute methanol (20 ml). The papers are dried at room temperature for 50 minutes.

Separation of lobeline
Spots of lobeline solution or lobelia alkaloids in chloroform are applied on the starting lines of the above chromatograms and these are developed with the solvent chloroform-benzene (9:1) saturated with formamide. After developement the chromatograms are dried.

Colorimetric determination
The spots corresponding to lobeline are cut out and shaken for 3 minutes in a stopper tube with 2 ml of buffered tropaeolin OO solution (pH 3.4) and 5 ml chloroform. The resulting mixture is centrifuged for 2 minutes. An aliquot of the chloroform layer is measured using a suitable filter photometer.
A blank is treated similarly and the lobeline content is then read from a caliberation curve. The mean error is ± 2.0% (75).

Further two colorimetric methods have been described for the determination of lobeline and the other alkaloids of lobelia. These methods depend on formation of a dye complex with alkaloids and measuring this complex at certain wavelengths (76,77).
In the first method (76), lobeline (<0.5 mg) is extracted from paper chromatography (which used for its separation) with citrate-phosphate buffer of pH 7.5 (20 ml), 0.15% Bromothymol blue solution (1 ml) is added and the mixture is then extracted with chloroform (3x20 ml). The combined chloroform extracts are mixed with 2% ethanolic boric acid (25 ml). The resulting mixture is filtered, diluted to 100 ml with ethanol and its extinction is measured *at 436 nm* against a reagent blank in 2-cm. cell.
The reproducibility ± 2% (76).
In the other method, lobeline and the total alkaloids of lobelia and its preparations can be determined by almost similar technique (77).
The powdered drug is extracted by shaking with a mixture of methanol - 0.1 N HCl (1:1) and centrifuging.
An aliquot of the supernatant liquid (neutralized with NaOH solution) or of lobeline injection is mixed with 0.01% methyl orange in Mc-Ilvaine buffer solution (pH 5.0) and the resulting dye complex-alkaloids are extracted in chloroform. The chloroform extract is shaken with 0.1 N HCl containing 5% NaCl and the absorbance of the liberated dye in the acid solution is measured *at 510 nm*.
Lobeline remains in the chloroform layer (as its HCl salt) and this layer is treated with more dye solution then the mixture is extracted with 0.1 N HCl and this acid extract contains dye equivalent to the lobeline. The extinction of this is also measured at the same wavelength. Beer's law is obeyed for 0.04 to 0.7 mg of lobeline. The coefficient of variation of the method is 1.24% (77).

Other colorimetric method for lobeline determination has been reported (78).

8.6.2 UV Determinations

Lobeline was determined in ampoules, ethereal lobelia tincture and powder lobelia by charge-transfer complexation technique (79). Separate portions of lobeline extracts were transferred into two flasks (A & B), 5 m M-iodine (2 ml) was added to flask A and the contents of both flasks were made up to 20 ml with chloroform.
The absorbance of the solution in flask A was measured first *at 292 nm* against a blank of 0.4 m M-iodine in chloroform and again *at 292 nm* against the solution in flask B to obtain a difference reading.
Beer's law was obeyed for 1.6 to 9.6 mgl^{-1} (79).

— Lobeline hydrochloride injections are assayed by a UV technique (17):
An accurately measured volume (equivalent to about 0.002 g of lobeline HCl) is diluted with a buffer solution (prepared by dissolving 3.4g of sodium acetate in 5 ml acetic acid and sufficient water to produce 250 ml, pH 4.7) to produce 100 ml. The absorbance of an aliquot of this is determined at *248 nm* and the content of lobeline HCl is then calculated using lobeline HCl as a standard reference.

8.6.3 Turbidimetric Determinations

Lobeline in biological fluids *(urine and blood serum)* has been determined by a spectrophotometric method (80).
The method is as follows:
To a urine sample (10 ml), mercuric chloride (0.1 g) and 2 M NaOH (1 ml) are added. The mixture is then filtered through a filter paper. 10% HCl in anhydrous acetic acid (2 ml) is added to the filtrate, and the mixture is thoroughly mixed. To an aliquot of the mixture (5 ml), Nessler reagent (0.2 ml) is added and after 20 minutes, the turbidity so produced is measured *at 580 nm* against a blank.
Beer's law is obeyed with 2 to 20 μg of lobeline per ml biological fluid.
The mean error of the procedure is ± 1.2% (80).

8.7 Chromatographic Methods

8.7.1 Paper Chromatography

*Clarke (19) described the following technique for the identification of lobeline. This technique was originally deviced to screen nitrogenous bases (*81,82).
Whatman No.1 sheets 14x6 inches, were buffered by dipping in 5% solution of sodium dihydrogen citrate, blotting and drying at 25° for 1 hour, it can be stored indefinitely.
A solvent composed of 4.8 g citric acid in a mixture of 130 ml water and 870 ml n-butanol was employed. Lobeline exhibited Rf value of 0.82.
Lobeline was visualized with one of the followings:

- Examination under UV (254 nm), gives strong absorption.
- Spraying with iodoplatinate reagent.
- Spraying with Dragendorff's reagent.

A solvent system consisting of chloroform/benzene (9:1) was used to separate and identify lobelia alkaloids on paper chromatography. Lobeline exhibited Rf value of 0.78 in this system (83).

Other systems for paper chromatography have also been reported (75,84).

Quantitative determination of lobeline hydrochloride (as 0.2 mg injections) was performed on paper chromatography (85). Lobeline was produced as spots on 0.3 Cationite paper and after developement, spot were measured by a planimeter. A standard error of ± 2.06 was reported.

8.7.2 Thin Layer Chromatography (TLC)

Many TLC techniques were reported for the fast and reliable identification of lobeline and other lobelia alkaloids. Several of these are presented in the following table 7.

Table 7 : Thin Layer Chromatography of Lobeline

Chromatogram	Solvent System	Rf	Refer.
1. Silica gel G (0.25 mm layers)	Methanol-strong ammonia (100:1.5)	0.55	(19,86)
2. Silica gel G layers dipped in or sprayed with 0.1 M potassium hydroxide in methanol and dried	Methanol-strong ammonia solution (100:1.5) Cyclohexane-toluene-diethylamine (75:15:10) Chloroform-methanol (90:10)	0.61 0.17 0.35	(22)
3. Silica gel G layers	Chloroform-acetone-diethylamine (5:4:1)	0.68	(87)
4. " "	Chloroform-diethylamine (9:1)	0.90	(87)
5. " "	Cyclohexane-chloroform-diethylamine (5:4:1)	0.48 0.63	(87) (88)
6. " "	Cyclohexane-diethylamine (9:1)	0.14	(87)
7. " "	Benzene-ethylacetate-diethylamine (7:2:1)	0.48	(87)
8. Basic silica gel	Methanol	0.55	(87)
9. Aluminum oxide layers	Chloroform	0.55	(87)
10. " "	Cyclohexane-chloroform (7:3) - 0.05% diethylamine (3 drops/100 ml)	0.60	(87)
11. Kieselgel (silica gel) F_{254}	Methanol-25% ammonia (100:1.5)	1.25 relative to bupranolol	(89)

Other TLC have also been reported (90,91).

8.7.3 Gas Liquid Chromatography (GLC)

Few GLC systems have been described to identify and determine lobeline. The following are these systems:

System I (22,92)

Column Condition:	2.5% SE-30 on 80-100 mesh Chromosorb G (acid washed and dimethyldichlorosilane treated) 2m x 4mm internal diameter glass column. Column temperature 100-300°.
Carrier Gas:	Nitrogen at 54 ml/minute
Reference:	n-Alkanes with an even number of carbon atoms.
Identification:	RI (Retention Index) for lobeline 1780.

System II (89)

Column Condition:	Glass column 6 ft. x 2 mm internal diameter. Packed with 3% OV-1 on Chromosorb W-HP 100-120 mesh. Column temperature 150-250°, programmed at 10°c/minute.
Carrier Gas:	Nitrogen at 50 ml/minute; air 120 ml/minute; hydrogen 2 ml/minute.
Detector:	N-FID.
Reference:	2-amino-5-chlorobenzophenone.
Identification:	Rel. t_R (relative retention time) for lobeline 0.71. RI 1823.

System III (89)

Column Condition:	Glass column 6 ft. x 2mm internal diameter. Packed with OV-17 on Chromosorb W-HP, 100-120 mesh. Column temperature 150-250°c, programmed at 10°c/minute.
Carrier Gas:	Nitrogen at 50 ml/minute; air 120 ml/minute; hydrogen 2 ml/minute.

Detector: N-FID.

Reference: Methagualone.

Identification: Rel. t_R (Relative retention time) for lobeline 0.6. RI 2078.

8.7.4 High Performance Liquid Chromatography (HPLC)

For the separation, identification as well as quantitation of lobeline, few HPLC systems were reported and these are as follows:

System 1 : The following system 1 has been employed to identify and quantitatively determine lobeline hydrochloride in antismoking chewing gum (93). The method depends on degrading lobeline into acetophenone and determine the latter.

Procedure: To chewing gum (1 g), aqueous 95% ethanol (20 ml), 1 M KOH (10 ml) and water (10 ml) are added. The resulting mixture is heated under reflux for 4 hours and is then distilled after adding water (100 ml). The distillate is diluted to 100 ml with water. An aliquot of 10 µl of this is analyzed by the following HPLC system.

Column: Spherisorb ODS (10 µm).

Mobile phase: Methanol-water (1:2).

Detection: UV at 249 nm.

The caliberation graph was rectilinear for 0.03 to 0.09% of lobeline in the sample.

System 2 : This system is used to identify lobeline (89).

Apparatus: A Perkin Elmer LC-series 312.

Column: RP-18, 10 µm, 250x4 mm^2.

Mobile phase: Acetonitrile (Lichrosolv), flow rate 1 ml/minute.

Reference: MPH = 5-(p-methylphenyl)-5-phenylhydrantoin.

Detection: UV at 220 nm.
Rel. t_R 0.73

System 3 : This system is also employed for the identification of lobeline (89).

Apparatus: A Perkin Elmer LC-series 312.

Column: RP-18, 10 µm, 250 x 4 mm^2

Mobile phase: 156 g acetonitrile + 344 g phosphate buffer (4.8 g 85% H_3PO_4 and 6.66 g KH_2PO_4) pH 2.3. flow rate 1 ml/minute (isocratically).

Detection: UV at 220 nm

Reference: MPH.
Rel. t_R 0.77

8.7.5 Ion Exchange Resins

This technique is used for separation and purification of organic substances as well as for quantitative determinations of these substances.

- An ion exchange method was used for the quantitative analyses of several alkaloids including lobeline (94). The solution of lobeline hydrochloride passed through a column (14 cm x 1 cm) packed with Wofatit KPS (Zn^{2+} form). The Zn^{2+} in the eluate was determined with 5mM EDTA in the presence of Eriochrome black T in an ammonia buffer medium of PH 10.4.
- Alginic acid after treatment with formaldehyde was used as carboxylic cation exchange medium. The absorption from aqueous solutions and subsequent elution with ammonium sulfate solution followed by spectrophotometric determination of several alkaloids including lobeline were performed (95).

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ACKNOWLEDGEMENT

The author would like to thank Mr. Uday C. Sharma, Dept. of Pharmacognosy, College of Pharmacy, Riyadh, Saudi Arabia for his valuable and sincere efforts in typing this manuscript.

ANALYTICAL PROFILE OF LOMUSTINE

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Saudi Arabia.

Content

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

(a) 1-(2-chloroethyl)-3-cyclohexyl)-1-nitrosourea (1,2,3,4,5).

(b) N-(2-chloroethyl)-N′ -Cyclohexyl-N-nitrosourea (1,4).

(c) Urea N-(2-chloroethyl)-N′ -cyclohexyl-N-nitroso-urea (5).

1.1.2 Generic Names

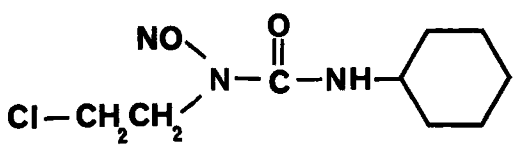
CCNU(1,5),NSC 79037(1,4).RB 1509(1,4),Belustine(1). CeeNU(1,5). Lucostine (2). Lomustine.

1.2 Formulae

1.2.1 Empirical

$C_9H_{16}ClN_3O_2$

1.2.2 Structural



1.2.3 CAS Registry Number

13010-47-4 (4).

1.3 Molecular Weight

233.69

1.4 Element Composition

C.	46.25%	H.	6.90%	CI.	15.18%
N.	17.98%	O.	13.69%		

1.5 Appearance and Color

A yellow crystalline powder (1,2,3,4,5).

2. Physical Properties

2.1 Melting Range

About 89°C (2,4). 90°C (1).

2.2 Solubility

Practically insoluble in water(2,3,5) very slightly soluble in water (1 mg/20 ml) (4) freely soluble in chloroform and soluble in ethanol and acetone(3,4) and highly soluble in lipid (5,6), also soluble in saline (7,8).

2.3 Stability

Drug solutions are stable for 7 days at 0°C (2). Commercially available lomustine capsules should be stored in well closed containers at a temperature less than 40°C preferably between 15-30°C. Lomustine capsules are stable for at least 2 years when stored in well closed containers at room temperature(6).The bulk drug should be stored in a deep freeze and protected from moisture(1).

2.4 X-Ray Powder Diffraction

The X-Ray diffraction pattern of Lomustine was determined on a Philips X-Ray diffraction spectrogoniometer equipped with PW-1730/10 generator (8). Radiation was provided by a copper target (cu anode 2000W, γ = 1.5418A) and high intensity X-ray tube operated at 40 KV and 35MA. Divergence slit and he

Table 1: X-ray diffraction pattern of lomustine

2θ	d(Å)	[I/Io(%)]	2θ	d(Å)	[I/Io(%)]
5.371	16.6068	4.6125	40.623	2.2208	0.607
7.346	12.0342	1.611	40.929	2.2049	0.511
8.222	10.7534	100	41.865	2.1578	0.690
11.550	7.6617	0.552	42.092	2.1467	1.894
13.088	6.7645	0.523	42.921	2.1071	0.802
16.244	5.4566	1.695			
1.513	5.3682	16.60			
17.480	5.0734	0.501			
18.342	4.8368	6.071			
19.296	4.5998	0.370			
21.582	4.1174	1.010			
22.154	4.0123	0.692			
22.416	3.9662	2.349			
23.187	3.8359	4.062			
24.056	3.6993	0.433			
24.279	3.6659	0.554			
24.878	3.5789	4.814			
25.332	3.5158	1.644			
26.164	3.4058	4.848			
26.540	3.3585	0.558			
27.263	3.2710	0.763			
28.135	3.1716	0.342			
29.054	3.0733	1.468			
29.677	3.0102	0.886			
30.441	2.9364	0.334			
30.825	2.9006	0.307			
32.241	2.7764	0.486			
32.574	2.7488	0.346			
33.368	2.6852	0.256			
34.394	2.6074	0.550			
34.672	2.5871	0.287			
36.123	2.4864	0.380			
36.945	2.4330	0.435			
37.216	2.4159	0.621			
37.918	2.3728	1.870			
38.413	2.3434	0.380			
40.039	2.2519	0.374			

dA = Interplanner distance.

I/Io = Relative intensity based on the highest as 100.

receiving slit were 1 and 0.1 respectively. The unit was equipped with Philips PM 8210 printing recorder and digital printer.

The x-ray pattern of lomustine is presented in fig. (1). Interpplanner distance and relative intensity are listed in table (1).

2.5 Spectral Properties

2.5.1 Ultraviolet Spectrum

The UV spectrum of Lomustine in ethanol was scanned from 200 to 350nm using 4054 UV/VIS LKB spectrophotometer (8). It exhibited a maximum at 230 nm (Fig 2).

2.5.2 Infrared Spectrum

The infrared spectrum of Lomustine as KBr disc is presented in (Fig. 3) and is recorded on Perkin-Elmer infrared spectrometer model 5808. Frequency assignments for some of the characteristic bands are listed in table (2).

Table 2: Infrared Spectral Assignments for Lomustine.

Frequency (cm^{-1})	Assignment
3360	N-H stretch
2850-2960	$CH_2 = CH_2$
1703	C=O stretch
1534 1491	Characteristic vibrations of the cyclohexyl ring
1083	N-O-N linkage.

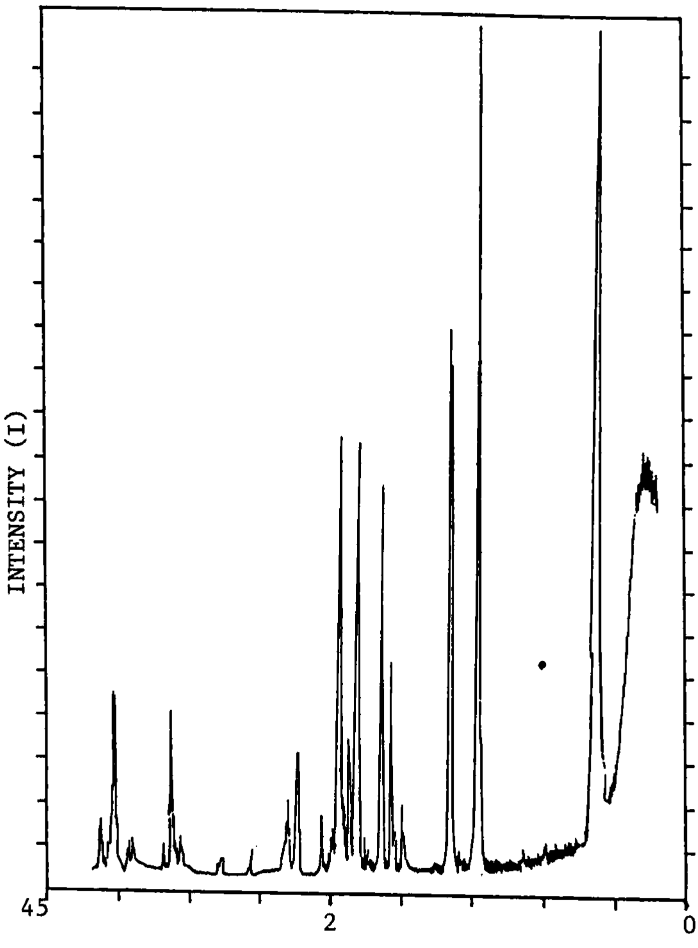


Fig. 1 The x-ray diffraction pattern of Lomustine.

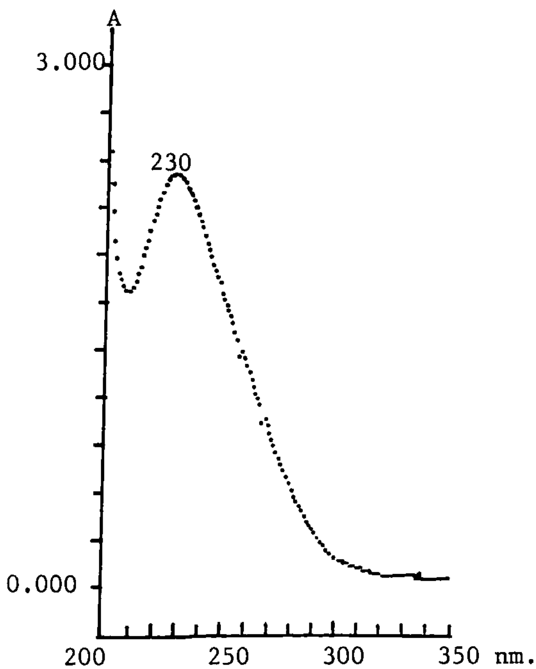


Fig 2. UV Spectrum of Lomustine in Ethanol.

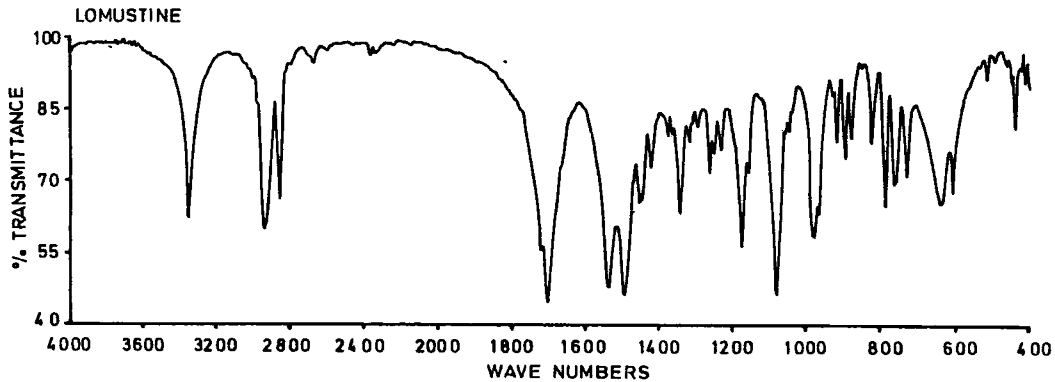


Fig. 3 I.R. Spectrum of Lomustine (KBr disc).

2.5.3 Nuclear Magnetic Resonance Spectrum

2.5.3.1 Proton Spectrum (PMR)

The PMR spectrum of lomustine in DMSO-d_6 presented in Fig (4), was recorded on a varian XL-200 NMR spectrometer using TMS as an internal reference. The structural assignments have been listed in table (3).

Table (3) PMR characteristics of Lomustine

Group	Chemical shift (ppm)
- CH_2	1.28 m
- CH_2 (cyclohexyl)	3.47 t
- C-H	3.49 m
N-CH_2	4.15 t
-CH_2	7.27 s

S= singlet, t= triplet, m= multiplet.

2.5.3.2 ^{13}C-NMR Spectra

The ^{13}C-NMR spectrum of lomustine in DMSO-d_6 using TMS as an internal reference is recorded on a Joel FX 100 FT NMR spectrometer (8) and is presented in Fig. (5).

2.5.4 Mass Spectrum

The mass spectrum of lomustine is presented in Fig. (6). This was obtained by electon impact ionization on a Finnigen 300 mass spectrometer by direct inlet probe at 270°C. The electron energy was 70 eV. The most prominent fragments their relative intensities and some proposed ion fragments are given in table (4).

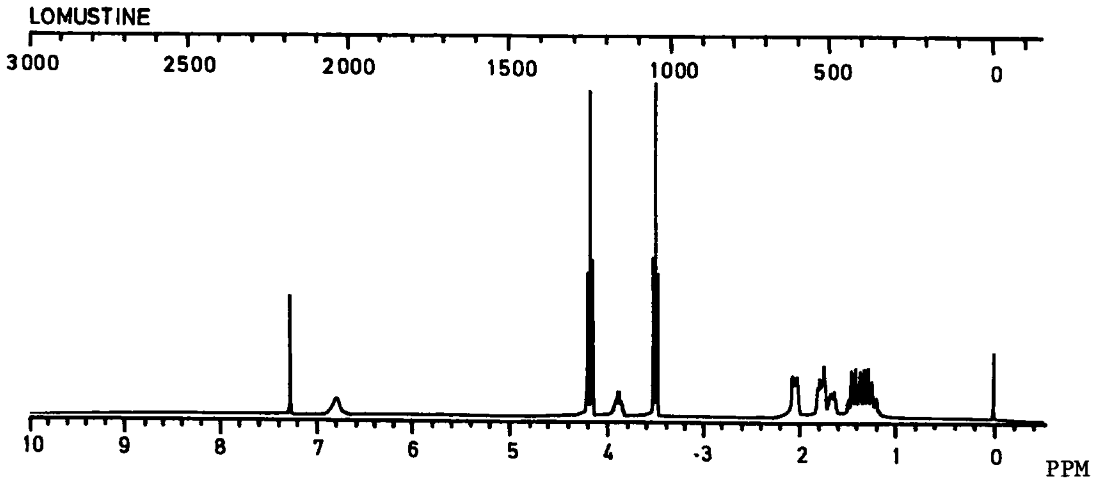


Fig. 4 PMR Spectrum of Lomustine in DMSO-d_6.

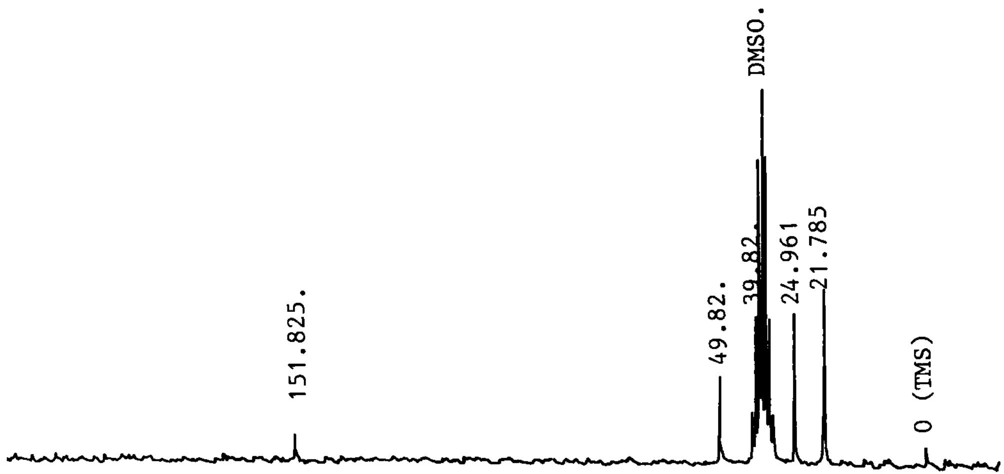


Fig. 5: ^{13}C-NMR noise decoupled spectrum of Lomustine.

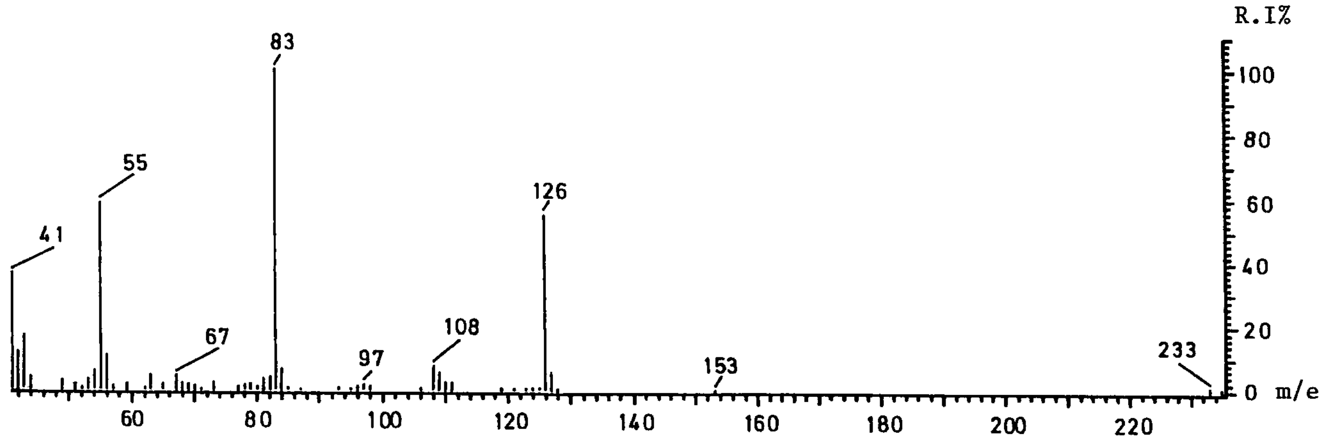
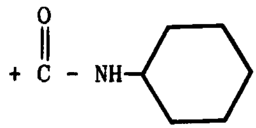
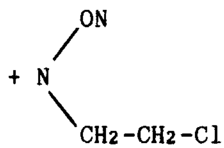
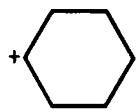


Fig. 6 Mass Spectrum of Lomustine.

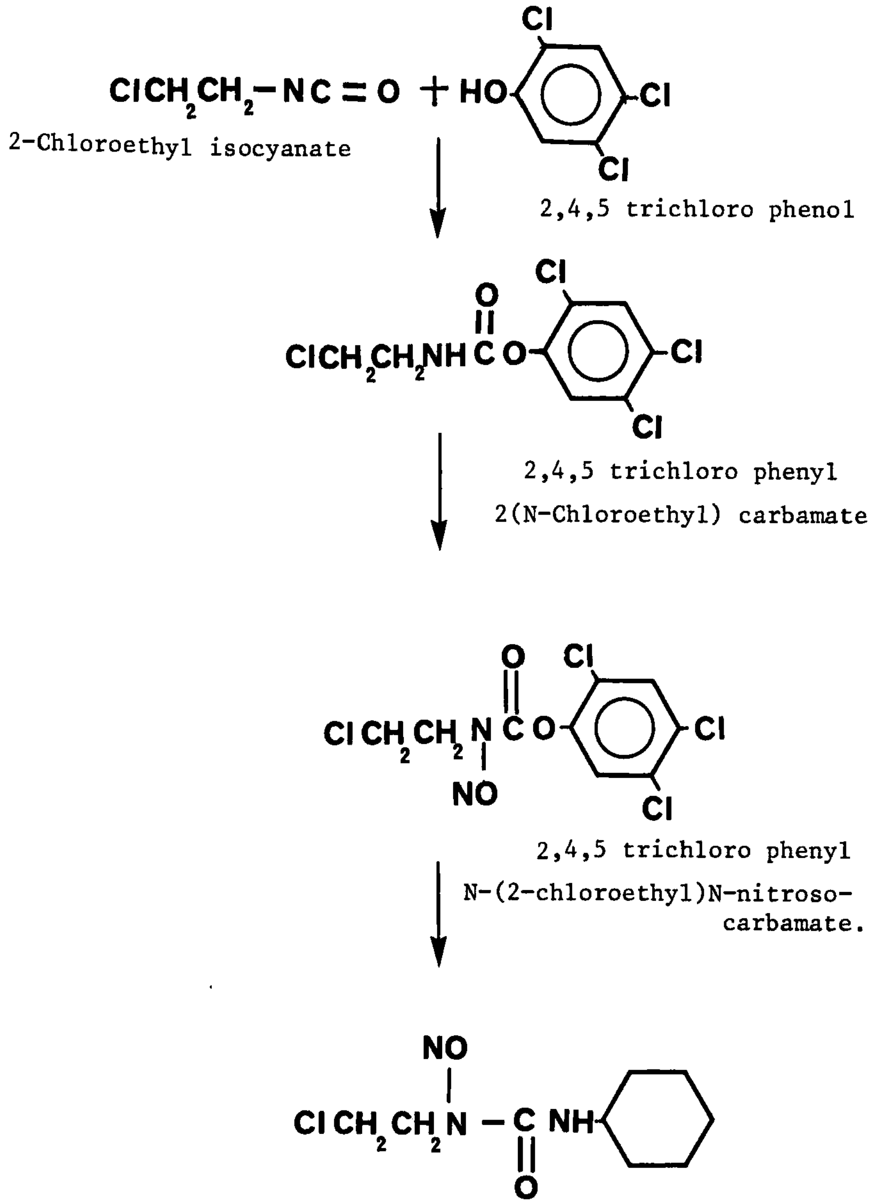
Table 4: Mass Fragments of Lomustine

m/e	Relative intensity%	Ions
233	2	M^+ (Lomustine)
126	56	
108	8	
83	100	
55	59	+ $CH-CH_2-N-N$
41	36	$N-CH_2-CH^+$

3. **Synthesis** (9)

A practical and convenient method for synthesizing antitumor compound (Lomustine) is based on the reaction of 2-chloroethyl isocyanate with 2,4,5, trichlorophenol to produce 2,4,5, trichlorophenyl-N-(2-chloroethyl)carbamate which on nitrosation with nitrosyl chloride in pyridine yeilds 2,4,5-trichlorophenyl-N-(2-chloroethyl) N-nitrosocarbamate as intermediate followed by the formation of N-(2-chloroethyl)-N-cyclohexyl-N-nitrosourea (lomustine). The product is isolated as crystalline, stable compound in very good yeilds.

Scheme:



N-(2-chloroethyl) N-cyclohexyl-N-nitrosourea.

(Lomustine)

4. Metabolism and Mechanism

Lomustine is absorbed (2) from the gastro-intestinal tract and is rapidly metabolised; metabolites have a prolonged plasma half life reported to range from 16-48 hours. The active metabolites readily appear in the crebrospinal fluid. About half a dose is excreted as metabolites in the urine within 24 hours but less than 75% is excreted within 4 days. About 60% of the cyclohexyl moiety of lomustine is reported to be bound to plasma proteins.

Lomustine and its metabolites (6) cross the blood brain barrier and are rapidly transported into cells due to their high lipid solubility. Drug is not detectable in CSF but active metabolites appear in substantial concentrations within 30 minutes after oral administration of lomustine CFS concentrations of metabolites have ben reported to be 15-50% or greater than concurrent plasma concentrations. Lomustine metabolites are present in milk, but in concentrations less than those in maternal plasma.

Lomustine is metabolized (6) in one hour after oral administration. The half life of lomustine metabolites is biphasic; although the initial plasma half life is 6 hours. The second phase plasma half life is 1-2 days and 15-20% of the metabolites remain in the body 5 days after administration of lomustine. Prolongation of plasma concentration is thought to reflect a combination of protein binding and enterohepatic circulation of metabolites.

It is distributed among the tissues with a volume of distribution greater than total body water. In the cerebrospinal fluid, the concentration of metabolites reaches 150% of that in plasma. Biotransformation occurs throughout the body, the half life is less than 1 hour.

Lomustine probably acts by a dual mechanism the cytotoxic effect involves the inhibition of DNA and RNA synthesis through alkylation and interference with histidine utilization, thereby upsetting the 1-carbon metabolic transfer process.

5. Uses.

Lomustine has been used (2) in the treatment of brain tumors, Hodgkin's disease, and also lung cancer, malignant melanoma and various solid tumors.

Clinical studies (6) of lomustine alone in the treatment of bronchogenic carcinoma, non-Hodgin's lymphomas, malignant melanoma, breast carcinoma, renal cell carcinoma, and carcinoma of the GI tract. Lomustine has been used topically in the treatment of psoriasis and mycosis fungoides.

Lomustine is given (10) by mouth to adults and children as a single dose of 130 mg per m^2 body surface, should be given to patients with compromised bone marrow function. Doses are also generally reduced when lomustine is given as a part of combination regimen. Providing blood counts have returned to acceptable levels, that is, platelets have 100,000 per mm^3 and leucocytes above 4000 per mm^3, doses may be repeated every 6 weeks, and should be adjusted according to the haemotological response.

6. Cautions and Adverse Effects

Lomustine is highly (6) toxic drug with a low therapeutic index. Nausea and vomiting occur in 45-100% of patients within 1-6 hours, after ingestion of an oral dose of lomustine. Thrombocytopenia and leukopenia reach (5) nadirs in 4 and 6 weeks respectively and last 1-2 weeks. Stomatitis, alopecia, anemia, and mild transient hepatotoxicity occasionally occur. Dysarthria, ataxia, lethargy and disorientation have been reported. Monitoring of leukocyte counts is required. When other myelosuppressive drugs are in use or have been used within the prior 4 weeks, the dose of lomustine should be reduced.

Other less frequently (6) reported adverse effects of lomustine include hepatotoxicity manifested by transient elevation of liver function test results and alopecia. A decrease in kidney size, progressive azotemia, and renal failure have occured in patients who received large cumulative dose after prolonged

therapy with lomustine and related nitrosoureas, renal damage has also occured occasionally in patients receiving lower total doses. A few cases of pulmonary infitrates and fibrosis, with onset occuring 6 months or longer after initiation of lomustine therapy and and cumulative doses of 600-1040mg have been reported-neurologic reactions including disorientation, lethargy, ataxia and dysarthria have been reported in some patients receiving lomustine. Adverse dermatologic effects resulting from topical application of lomustine for the treatment of psoriasis and mycosis fungoides including contact dermatitis, short term hyperpigmentation, long term telangiectasia, cutaneous pain, pruritus, and a Nikolsky-like epidermal separation in inflamed uninvolved skin. Adverse systemic myelosuppressive effects have also been reported following topical use of the drug.

7. Methods of Analysis

7.1 Elemental Analysis

C	46.25%	H	6.90%	Cl	15.18%
N	17.98%	O	13.69%		

7.2 Identification

a) The infrared absorbtion spectrum, is concordant with the reference spectrum of lomustine (3).

b) Carry out the test in subdued light and prepare the solution immediately before use. The light absorbtion, in the range 200 to 350 nm of 0.002% w/v solution in ethanol (96%) exhibits a maximum of 230nm. The absorbance at 230nm is about 0.52 (3)

c) To 0.2 g add 20 ml of a 20% w/v solution of potassium hydroxide and boil under a reflex condenser, for 2 hours. Add 75 ml of water and 4 ml of nitric acid, cool and titrate with 0.05M silver nitrate vs determining the end point potentiometrically. Repeat the operation without the substance being examined. The difference between the titrations represents the amount of silver nitrate required. Each ml of 005M

silver nitrate vs is equivalent to 0.01168g of $C_9H_{16}ClN_3O_2$ (3).

7.3 Spectrophotometric Method

For the analysis (11) of lomustine encapsulated in liposomes, after isolation of the drug containing liposomes from free drug, a portion of liposomes was mixed with an equal amount of conc HCl and heated at 100° until the dispersion was optically clear. The liquid was evaporated, and the residue was taken up in 2ml of acetate buffer solution (PH 4.62) and treated with 0.2-0.5 ml of a 0.5% solution of 4-(4-nitrobenzyl) pyridine in acetone. The mixture, in a sealed container, was heated in boiling water for 15-30 minutes and then cooled in ice. After diluting the filtrate to 5ml with acetone (avoiding exposure to light), 0.5ml of 0.5M-NaoH was added and 1 minutes later, the absorbance was measured at 450nm to determine the alkylating activity.

7.4 Polarographic Method

The method (12) involves the acid hydrolysis of lomustine which liberates HNO_2, an azo-dye is formed when a solution containing 0.01 to 0.04% of lomustine, 0.09% of sulphanilic acid 0.03% of N-1-napthylethylenediamine dihydrochloride in aqueous 40% dimethylformamide is heated for 5 minutes on a boiling water bath. The mixture is then cooled and absorbance is measured at 562 nm. The limit of detection is 0.4 to 06 µg/ml. A solution of 0.05M H_2SO_4 is optimum for the pulse polarographic determination of lomustine; the E valve is -0.475 V (vs. the s.c.e.). The limit of determination is 5 ng/ml.

7.5 Chromatographic Methods

7.5.1 Gas Chromatography

Different methods of Gas chromatography have been used for the determination of lomustine are summarised in the table (5).

Table 5: Summary of conditions used for GC lomustine

Column support	Mesh	Temperature	Low rate	Sample	Ref.
Glass column (1.5 m X 2 mm) packed with 3% OV-1.	100-120	--	40 ml/min helium carrier gas.	Plasma	13
(1.2 m X 2 mm) 10% of SE-30 on Gas-Chrom Q.	100-120	150°	--	Plasma	14
(1.2 m X 2 mm) packed with ultrabond 20 M	--	--	N_2 gas carrier gas	Plasma or urine	15
25-M fused-silica, SE 30 capillary column.	--	--	--	Plasma	16

7.5.2 Thin Layer Chromatography (TLC)

The separation of drug is carried out by TLC (3) using Merck silica gel and a mixture of toluene and glacial acetic acid (80.20) as the mobile phase. Apply seperately to the plate 4 µl of each of three freshly prepared solutions of the substance being examined in methanol, containing (1) 2-5% w/v, (2) 0.010% w/v and (3) 0.0050% w/v. After removal of the plate heat it at 110° for one hour, place the hot plate in a closed tank containing chlorine, produced by adding HCl to a 5% w/v solution of pot. Permanganate contained in a beaker placed at the bottom of the tank, and allow to stand for two minutes. Dry in a current of cold air until an area of the plate below the line of application produces at most a very faint blue colour with 0.05 ml of 0.5% w/v solution of potassium iodide in starch mucilage, avoid prolonged exposure to cold air. Spray the plate with a 0.5% w/v solution of potassium iodide in starch mucilage. Any secondry spot in the chromatogram obtained with solution (1) is not more intense than the spot in the chromatogram obtained with solution (2) and not more than one such spot is more intense than the spot in the chronatogram obtained with solution (3).

7.5.3 High Performance Liquid Chromatography H.P.L.C.

High performance liquid chromatography HPLC method (3) used to estimate lomustine is carried out by using stainless steel column (20 cm X 4mm) packed with stationary phase (10um) (Nucleosil C_{18}) and a mixture of equal volumes of methanol and water as a mobile phase with a flow rate of 2 ml per minute at the warelength of 230 nm.

7.6 Thermal Analysis (DSC)

A differential scanning calorimetry (8) curve was obtained Fig. (7) on a Perkin-Elmer DSC-2C differential calorimeter. Nitrogen was used as the purge gas scan was performed at a rate of 25°C/min from 60°-380°C. The DSC curve revealed an endothermic melting peak (Max. 91.79°C).

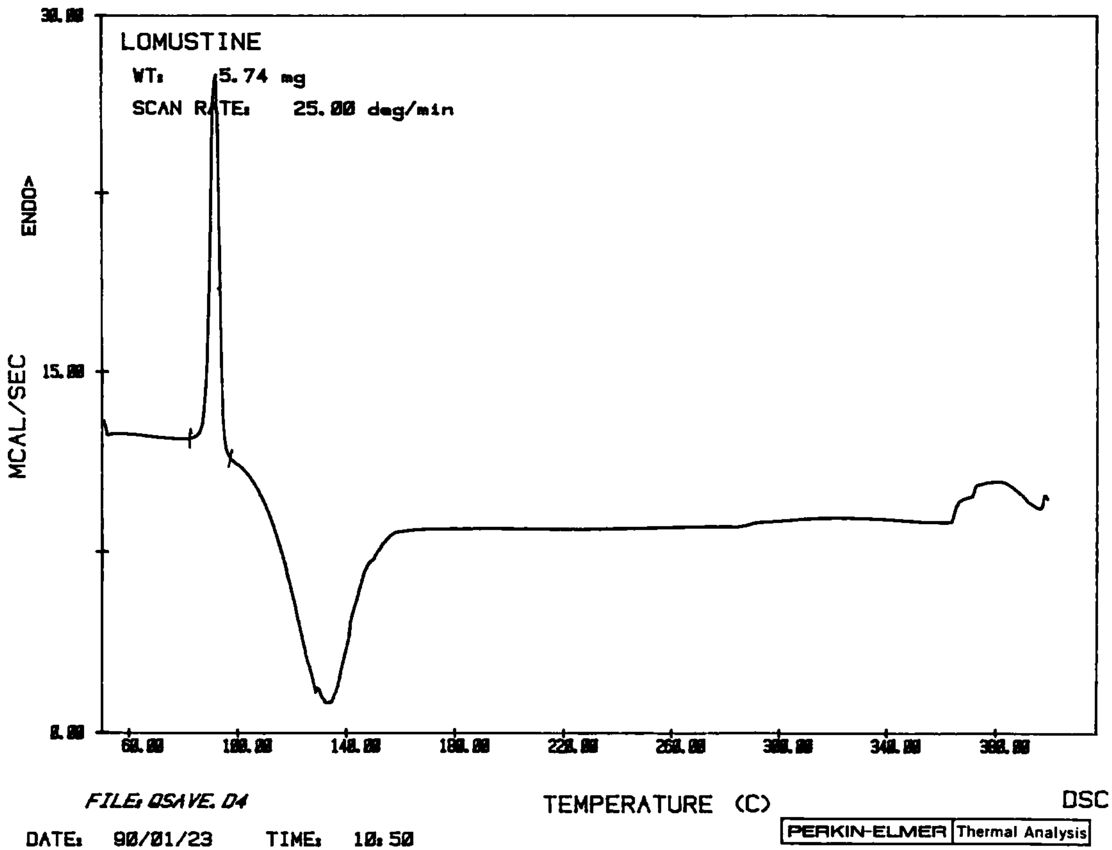


Fig. 7 Thermal curve of Lomustine.

8. Acknowledgements

The author is highly thankful to Mr. Tanvir A. Butt, Pharmaceutical Chemistry Department, College of Pharmacy, King Saud University, who extended a lot of efforts and taken great pains in typing the manuscript. The author also appreciates the technical services provided by Mr. Babkir Awad Mustafa of the College of Applied Medical Sciences, King Saud University, Riyadh, Saudi Arabia.

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LOPERAMIDE HYDROCHLORIDE

Jos Van Rompay and James E. Carter

TABLE OF CONTENTS

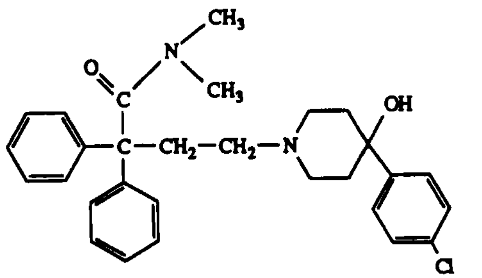
1. Description

1.1 Name, Formula and Molecular Weight

Loperamide hydrochloride or R 18553

*4-(4-chlorophenyl)-4-hydroxy-N,N-dimethyl-α,α-diphenyl-1-piperidinebutanamide monohydrochloride
CAS-34552-83-5. RINN, USAN, BAN

4-(p-chlorophenyl)-4hydroxy-N,N-dimethyl-α,α-diphenyl-1-piperidinebutyranamide monohydrochloride.



$C_{29}H_{33}ClN_2O_2.HCl$ M.W.477.04+36.46 = 513.50

1.2 Appearance

White to slightly yellowish crystalline powder.

1.3 History

Loperamide hydrochloride is an antidiarrheal synthesized in 1969 in the laboratories of Janssen Pharmaceutica, Beerse, Belgium. The synthesis of loperamide hydrochloride followed the synthesis of diphenoxylate hydrochloride (Janssen, 1956) as an antidiarrheal with less opiate like central nervous system (CNS) activity and the discovery of fentanyl citrate (Janssen, 1960) as a potent centrally acting narcotic analgesic with antidiarrheal activity. The dissociation of the opiate-like and antidiarrheal activities resulted in a very safe and effective antidiarrheal which today is marketed in 126 countries and was recently approved for over-the-counter marketing in the United States. Loperamide hydrochloride is an USP article[1] and is contained in the Merck Index[2]. The pharmacology and biochemical properties of loperamide hydrochloride have been reviewed[3].

2. Synthesis

The Janssen synthesis of loperamide hydrochloride is detailed in Figure1[4]. α-(2-Bromoethyl)-α-phenylbenzeneacetonitrile is converted through a series of reactions to N-[dihydro-3,3-diphenyl -2(3H)- furanylidene -N-methyl methanaminium bromide and ethyl 4-oxo-1-piperidinecarboxylate is converted to 4-(4 chlorophenyl) -4- piperidinol. The condensation of the basic piperidine nitrogen with furanylidene results in the formation of loperamide base which is then crystallized as the hydrochloride salt. Recrystallization has resulted in the identification of two polymorphs and a tetrahydrate form which will be detailed later. Although the patent on loperamide has expired and the product is available generically other syntheses for the compound have not been published.

3. Physical Properties

3.1 Infrared Spectra

The infrared spectrum of loperamide hydrochloride, polymorph I, polymorph II and a tetrahydrate form are presented in Figure 2[5]. All spectra were obtained as RbI pellets. The polymorphic forms are readily distinguishable through their IR spectra. The tetrahydrate contains the characteristic OH stretch centering at 3400 cm^{-1}. In the carbonyl stretch region polymorphs I, II and the tetrahydrate absorb at 1622 cm^{-1} , 1601 cm^{-1} and 1618 cm^{-1} respectively.

3.2 NMR Spectrum

The 200 MHz proton NMR Spectrum of loperamide base in $CDCl_3$ is shown in Figure 3[6]. The spectrum was obtained with a Bruker W.P. 200 Fourier Transform spectrometer equipped with an Aspect 2000 computer with the following instrument settings: sweep width 3521 Hz; pulse angle, 30°; pulse delay, 1 sec; data points, 32,000; measuring mode, quadrature - 8 pulse sequence; temperature, ambient. The structure with proton assignments is defined in Table 1.

3.3 Mass Spectrum

The mass spectrum of loperamide hydrochloride was obtained using a Varian MAT 311 instrument operating in the electron impact mode[7]. The spectrum was recorded using the direct inlet system at 160°C with a source pressure of 10^{-6} Torr. A current of 300 uA and energy 70 eV. The mass fragmentation

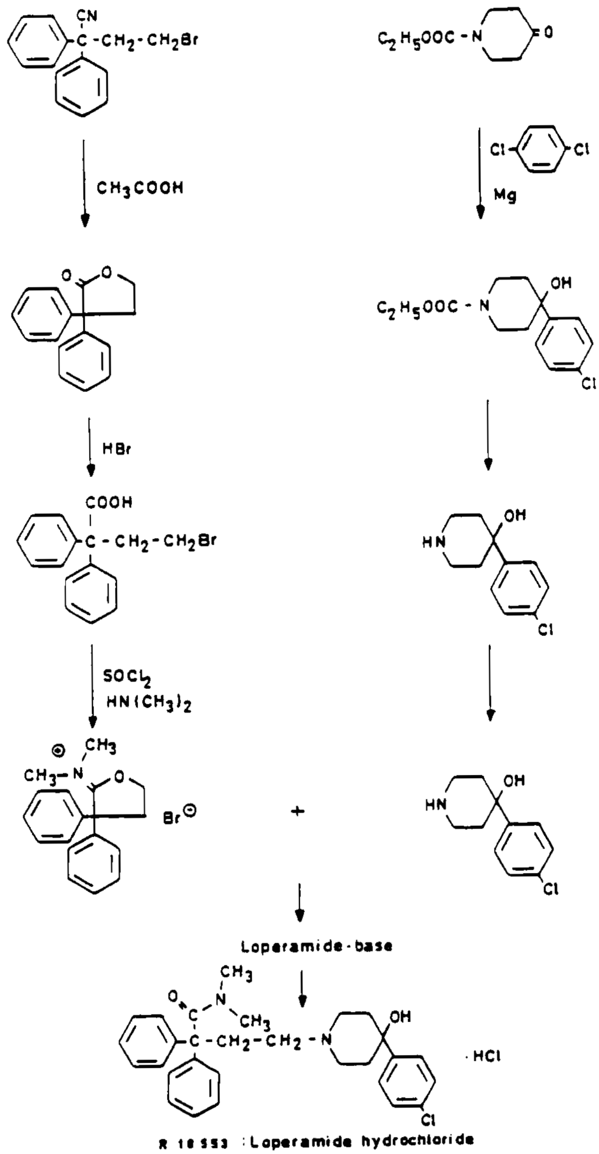


FIGURE 1. Synthetic Pathway to Loperamide Hydrochloride

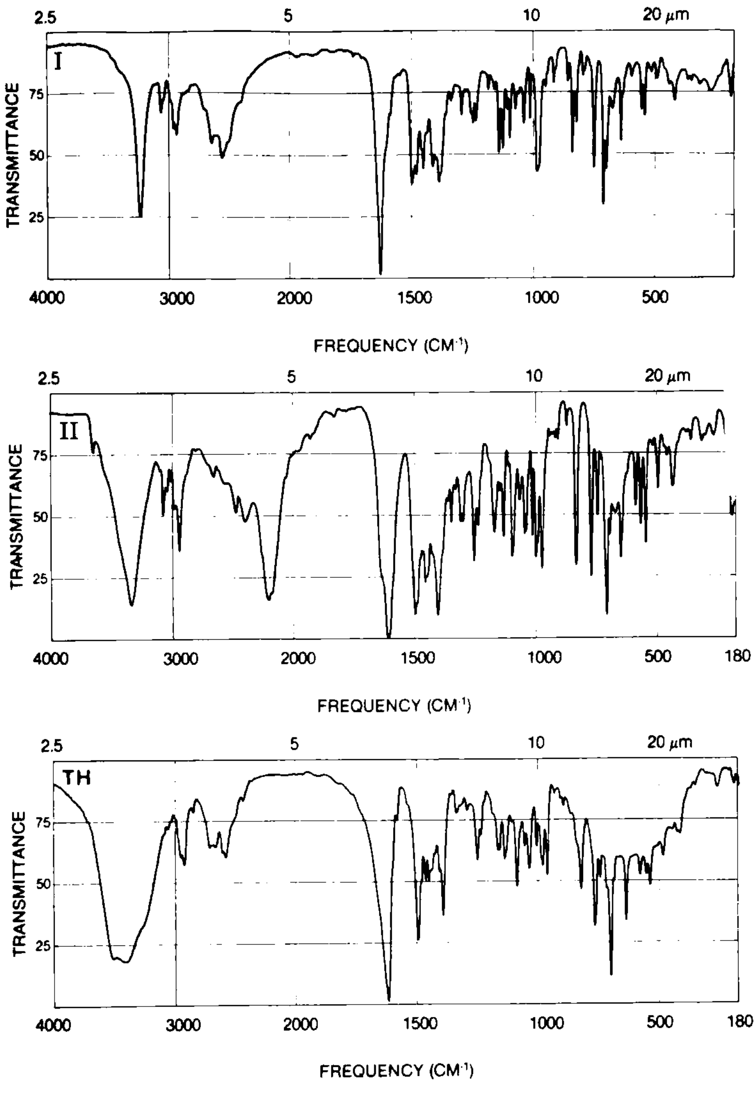


FIGURE 2. Infrared Spectra of Loperamide Hydrochloride Polymorph II and Tetrahydrate.
Instrument: Perkin-Elmer Model 580B

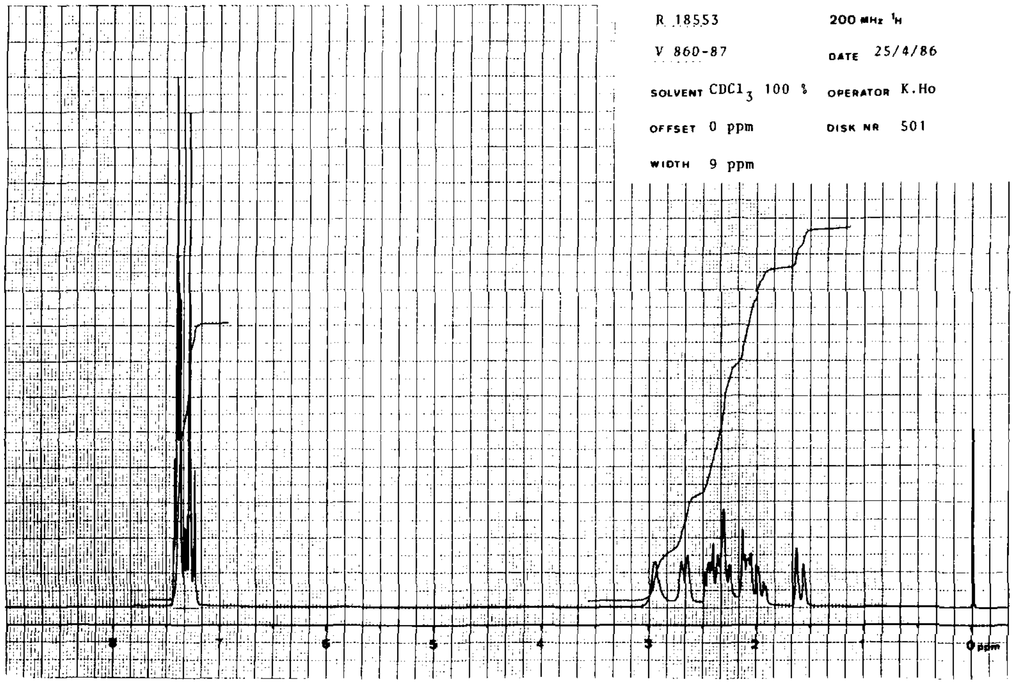
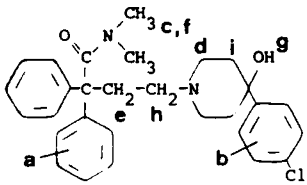


FIGURE 3. Proton 200 MHz NMR Spectrum of Loperamide Hydrochloride
Instrument: Bruker W.P. 200 Fourier Transform Spectrometer

Structure.



$C_{29}H_{33}ClN_2O_2.HCl$

Results.

Protons	δ(ppm)	Relative Surface	Multiplicity	J (Hz)
a, b	~7.2-~7.5	14	multiplet	
c	2.96	3	broad singlet	
d (eq)	2.68	2	multiplet	
e, d (ax), f	2.25-2.50	7	multiplet	
g, h, i (ax)	1.90-2.15	5	multiplet	
i (eq)	1.60	2	multiplet	

Table 1
Structure and Proton Assignments for 200 MHz NMR of Loperamide Hydrochloride

behavior showing some of the possible fragmentation ions is contained in Figure 4; the mass spectrum is contained in Figure 5. The proposed structure of the base peak containing chlorine at m/e 238 is supported by the peak at m/e 240 approximately one-third in size. Likewise, the structure of the strong peak at m/e 224 is supported by the peak one third in size at m/e 226.

3.4 Ultraviolet spectrum

The ultraviolet absorption spectrum of approximately 40 mg loperamide hydrochloride in 100 mL of 0.1 N hydrochloric acid/2-propanol (10/90,v/v) is shown in Figure 6[8]. The absorptions (detailed below) are the sum of the three substituted phenyl groups.

Maxima	
λ nm	ε
253	532
259	648
265	581
273	233

3.5 Melting Range

Loperamide hydrochloride (polymorph I) melts with decomposition at ~224°C[9].

Poymorph II~ 218°C
Tetrahydrate~ 190°C

3.6 Differential Scanning Calorimetry

The DSC curves of polymorphs I, II and the tetrahydrate are presented in Figures 7,8 and 9 respectively[10]. All curves were obtained with a Dupont 910 Differential Scanning Calorimeter and a Dupont 1091 data analyzer. The instrument was calibrated using an accurately weighed amount of pure indium under the same conditions used for sample measurement (melting point of pure indium: 156.6°C; heat of fusion: 28.4 J/g). From the DSC curves the melting point of

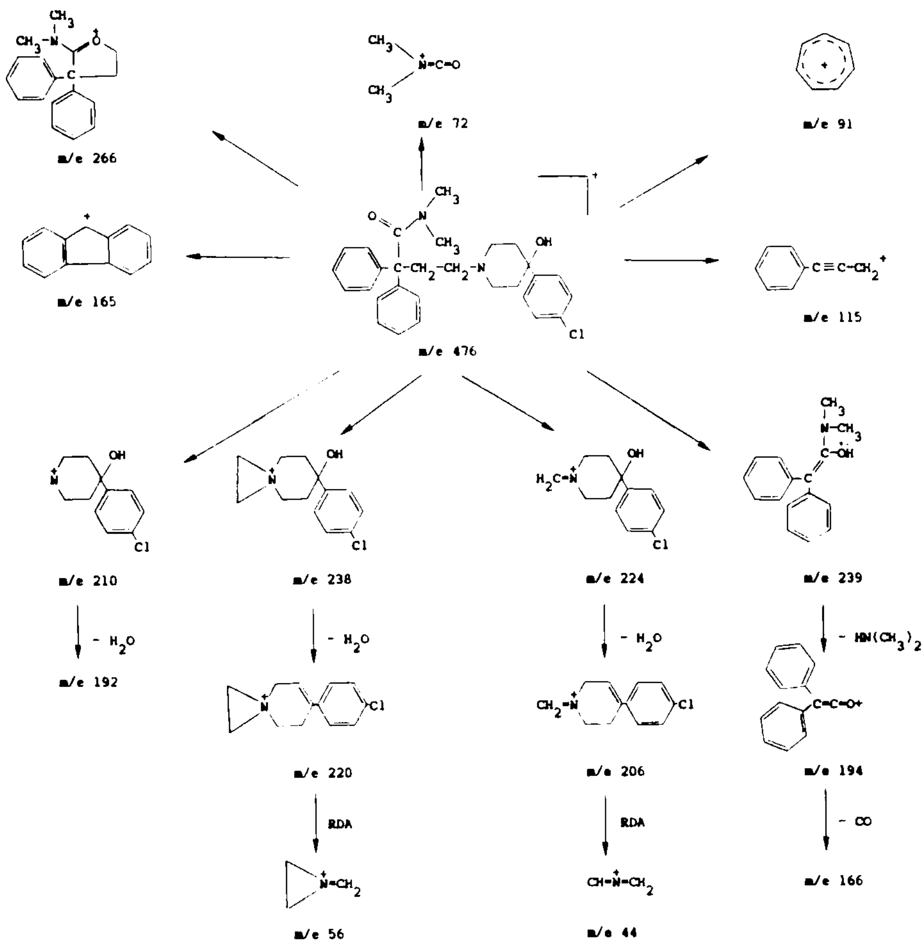


FIGURE 4. Mass Fragmentation Ions for Loperamide Hydrochloride

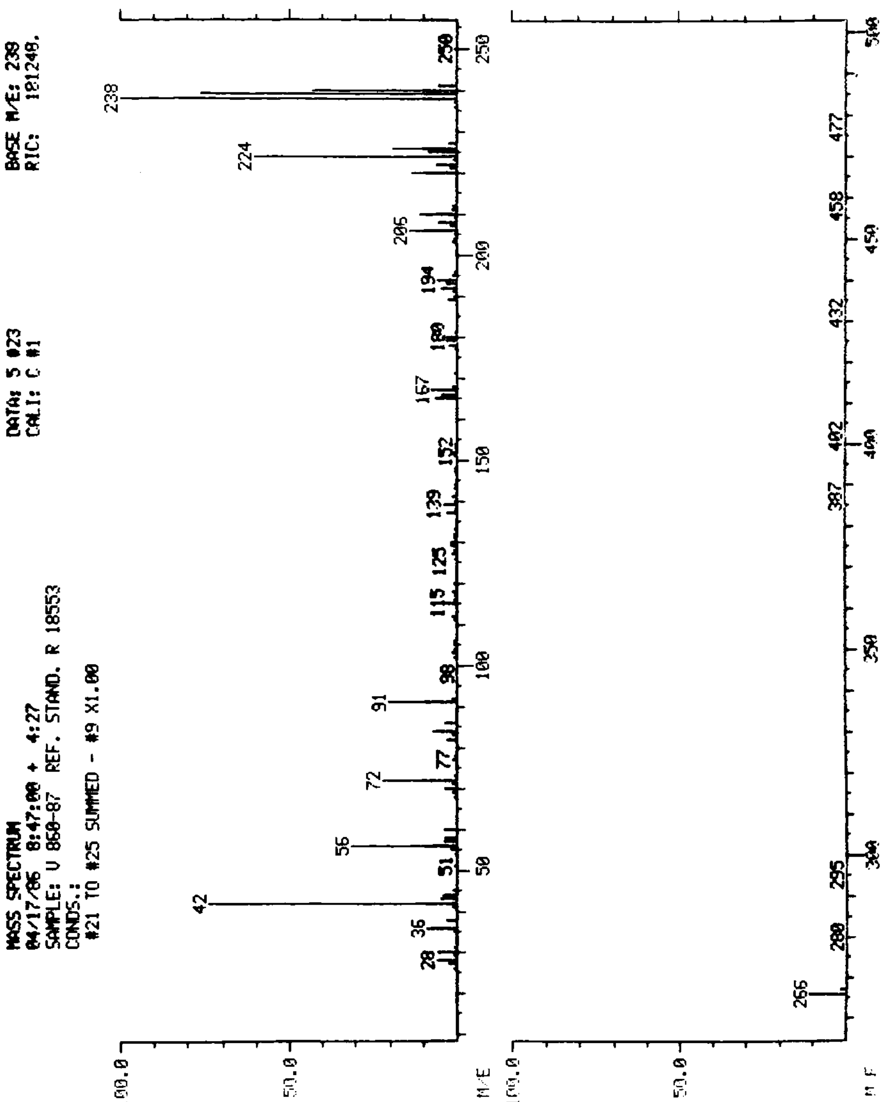


FIGURE 5. Electron Impact Mass Spectrum of Loperamide Hydrochloride
Instrument: Varian MAT 311

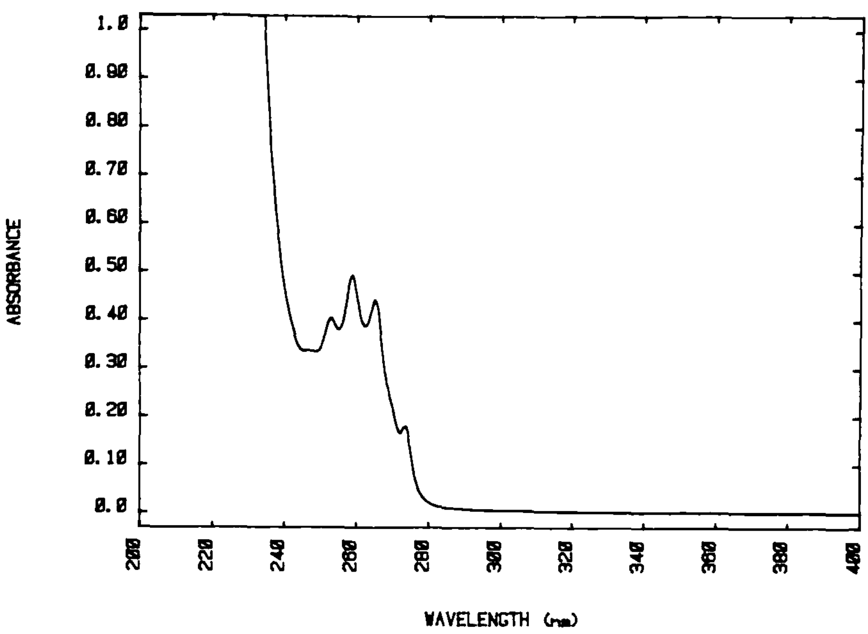


FIGURE 6. Ultraviolet Absorption Spectrum of Loperamide Hydrochloride
Instrument: Hewlett Packard 8450A

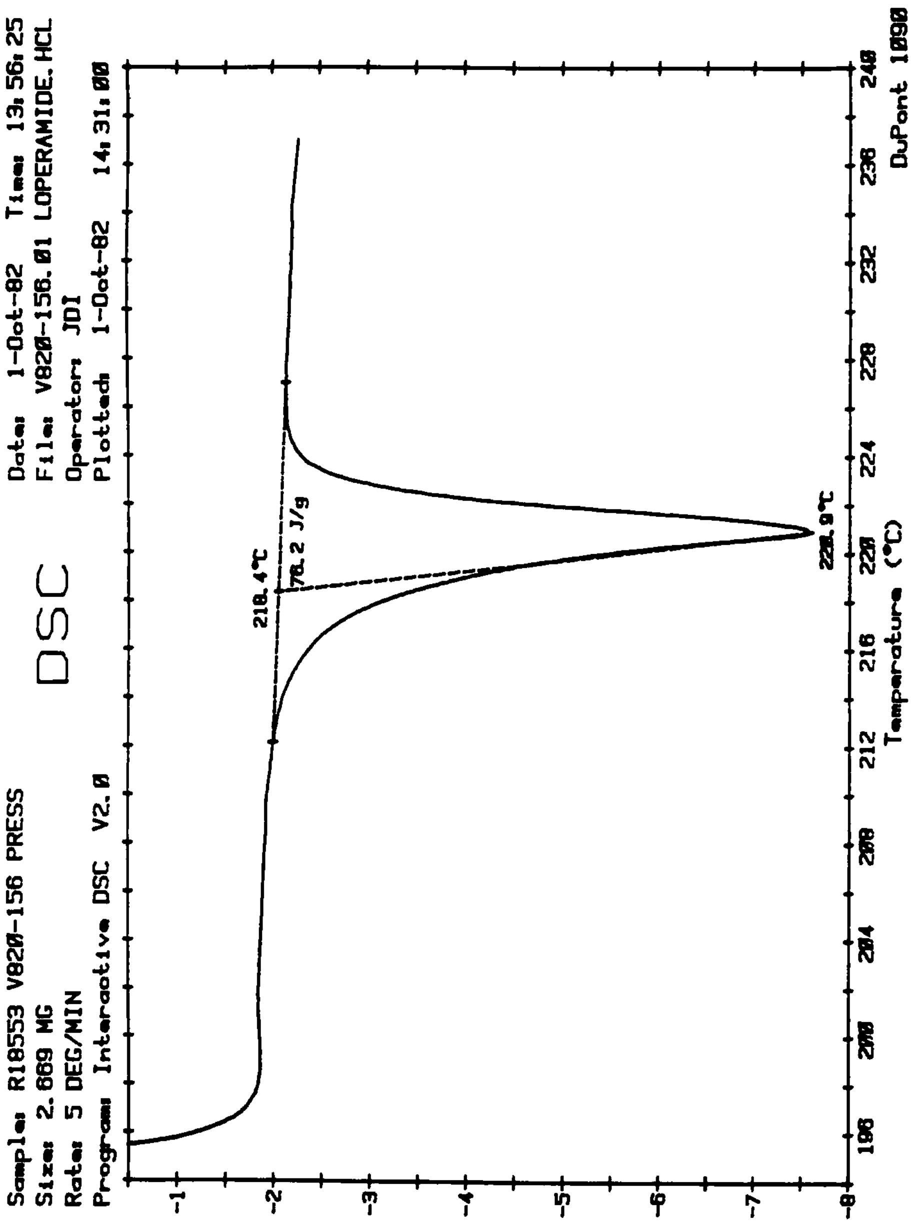


FIGURE 7. DSC Curve of Loperamide Hydrochloride Polymorph I
Instrument: Dupont 910

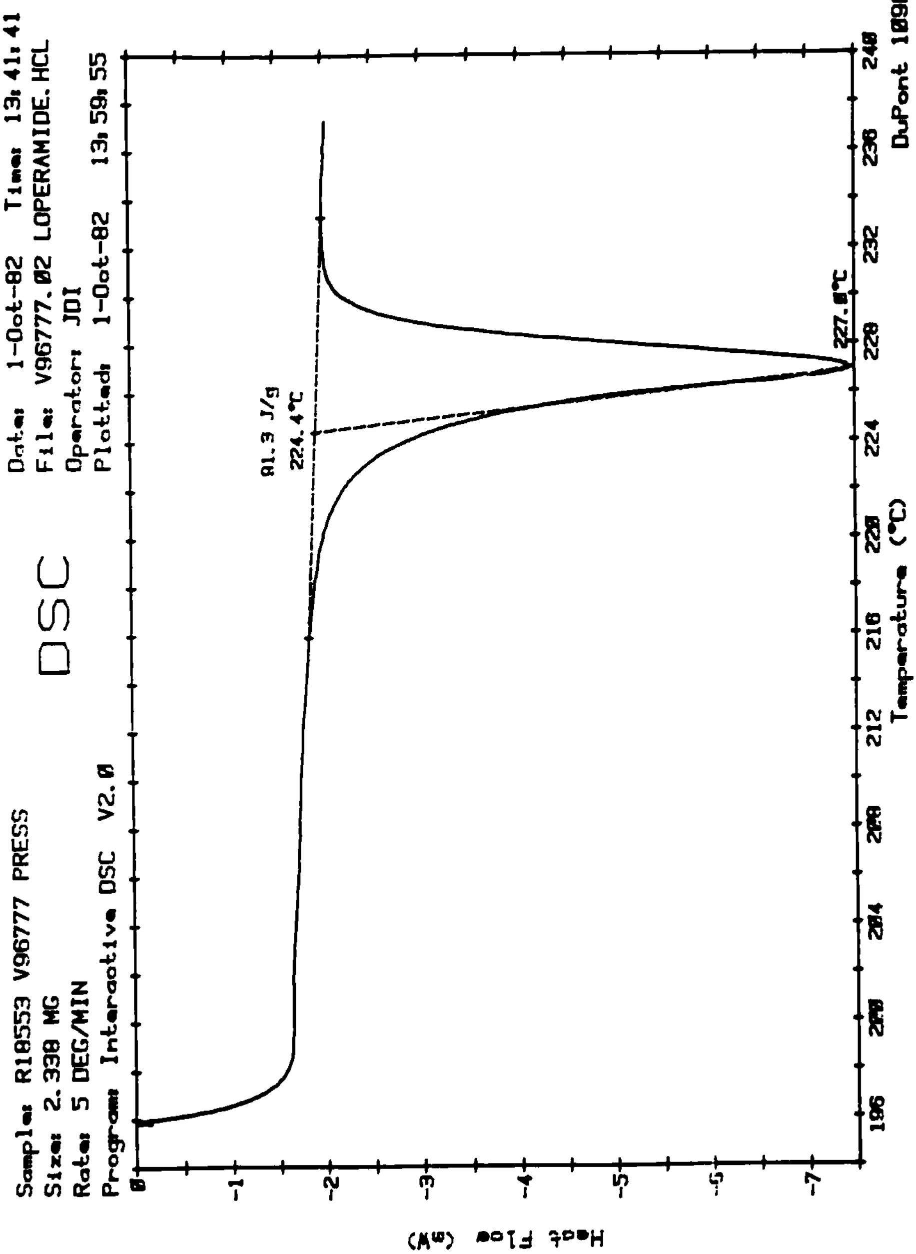


FIGURE 8. DSC Curve of Loperamide Hydrochloride Polymorph II
Instrument: Dupont 910

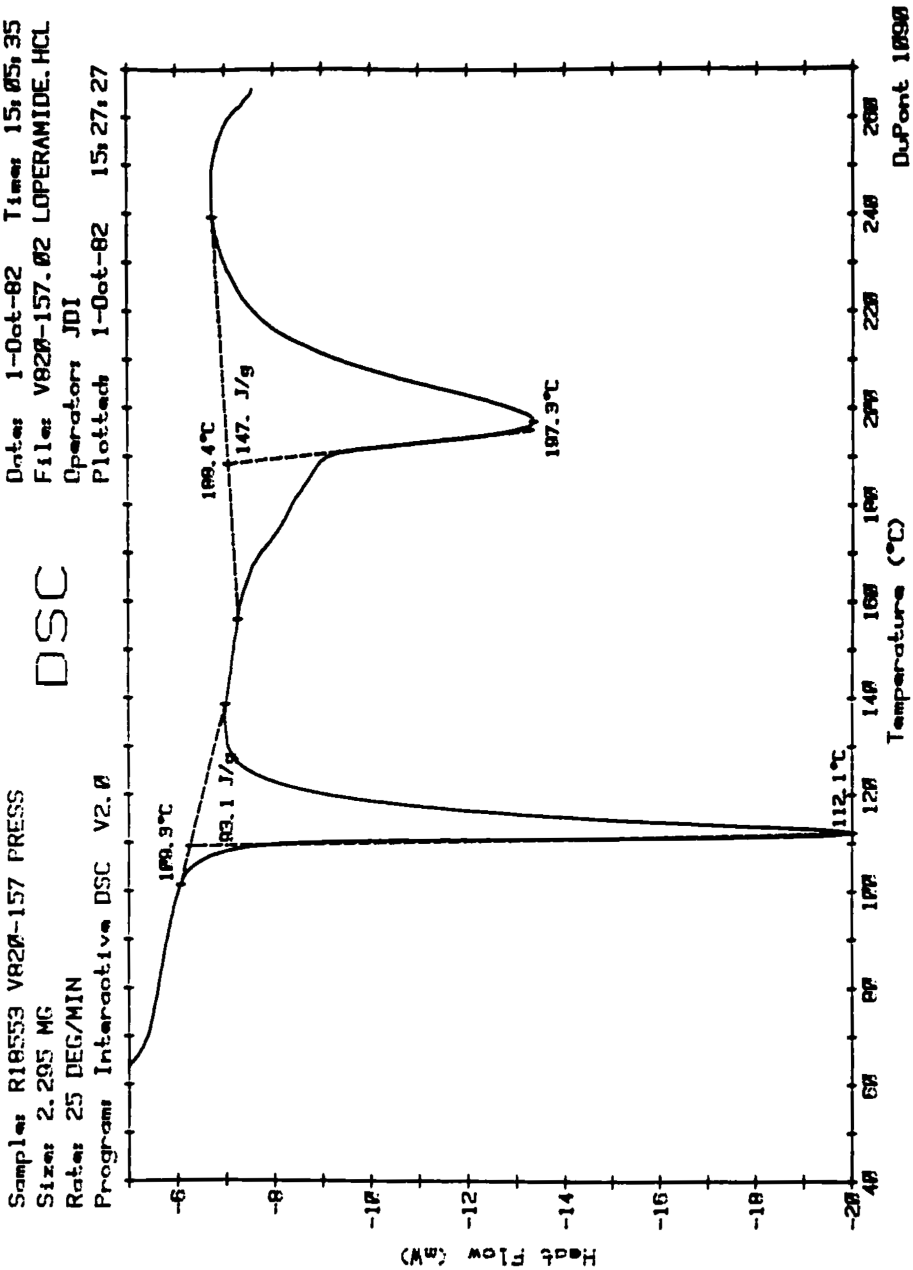


FIGURE 9. DSC Curve of Loperamide Hydrochloride Tetrahydrate
Instrument: Dupont 910

polymorph I is 224.4°C; the heat of fusion is 91.3 J/g. For polymorph II the melting point is 218.4°C; the heat of fusion is 76.2 J/g.

3.7 Thermal Gravimetric Analysis

TGA of the tetrahydrate form of loperamide hydrochloride was performed with a Dupont 1090 Thermal Analyzer at a heating rate of 20°C/min to a maximum temperature of 230° C[11]. Weight loss due to the evaporation of solvated water was 14.5%.

3.8 Ionization constant, pK

The ioniziation constant of loperamide hydrochloride was determined by potentiometric titration in methanol-water mixtures of varying composition and extrapolation of the values to pure water [12]. The ionization of the piperidine nitrogen results in a pKa of 8.66[13].

3.9 Solubility

The solubility of loperamide hydrochloride in various solvents is contained in Table 2[14]. The Merck index reports the solubility at physiological pH as 0.002%[2].

3.10 Crystal Properties (see also Sections 3.1, 3.5, 3.6)

Loperamide hydrochloride has been obtained in three crystalline forms; two distinct polymorphs and a tetrahydrate. Polymorph I is obtained by crystallization in 2-propanol, cooling with stirring, washing in ethylacetate and filtration. Polymorph II is obtained by cooling and spontaneous crystallization from 2-propanol without stirring. The tetrahydrate form was obtained from ethanol/water with cooling and stirring. X-ray diffraction patterns for polymorphs I and II and the tetrahydrate are presented in Figures 10, 11 and 12 respectively[15]. The patterns were obtained by the powder method using $CuK\alpha$.

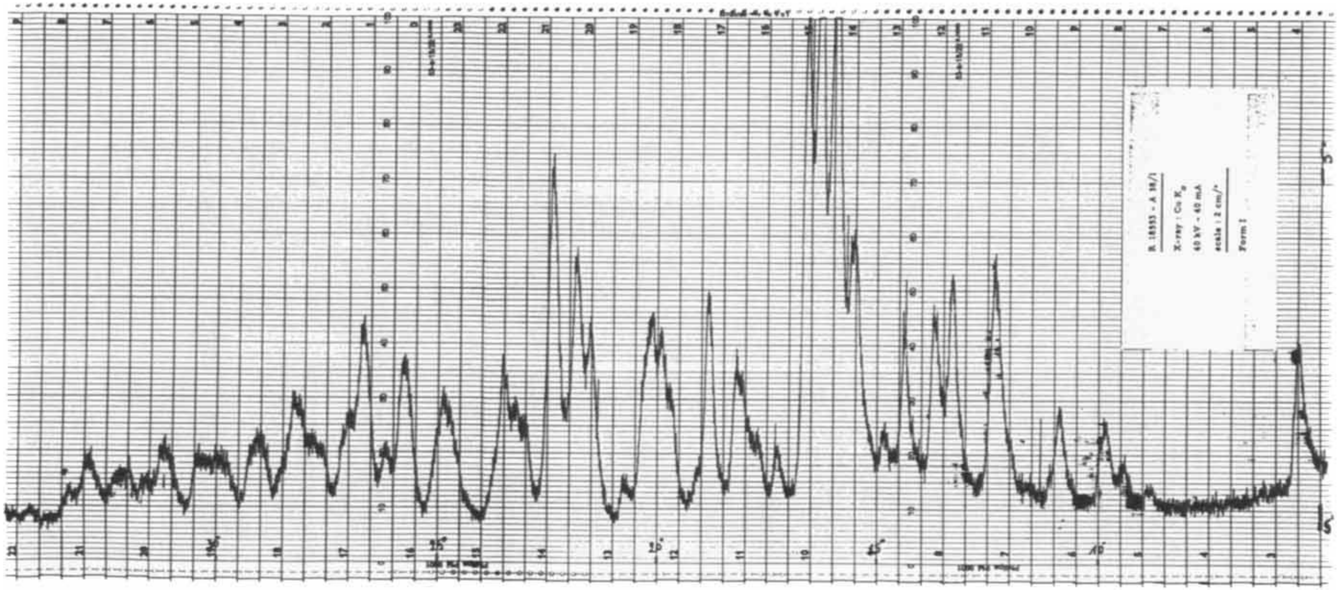


FIGURE 10. Powder X-ray diffraction pattern of Loperamide Hydrochloride Polymorph I

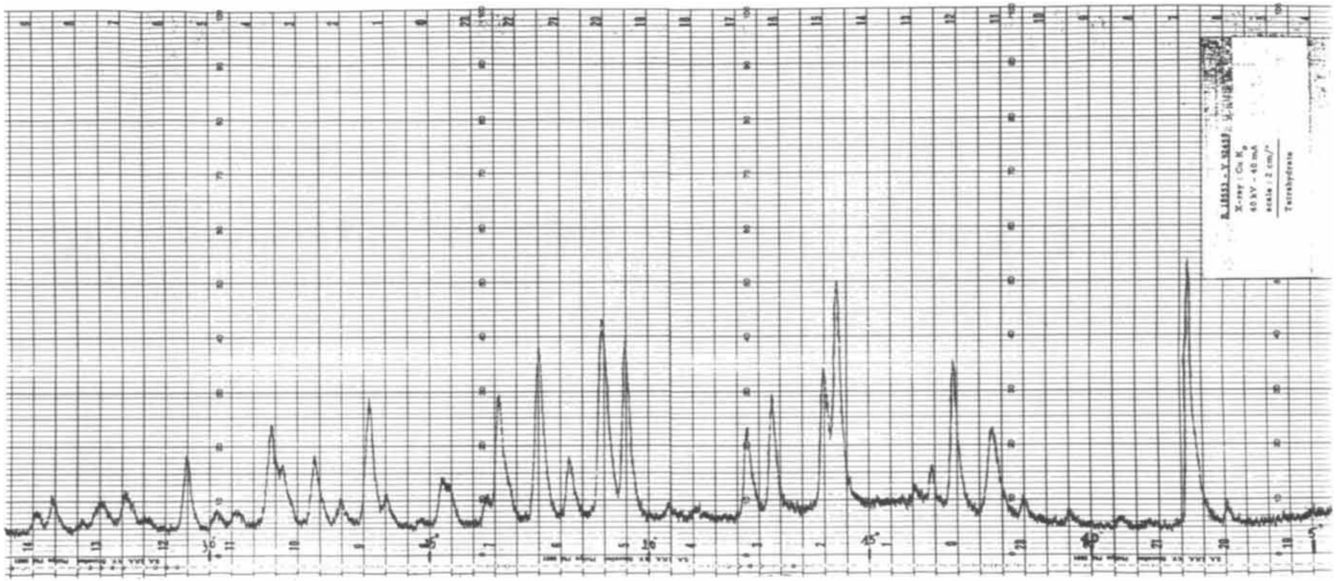


FIGURE 11. Powder X-ray diffraction pattern of Loperamide Hydrochloride Polymorph II

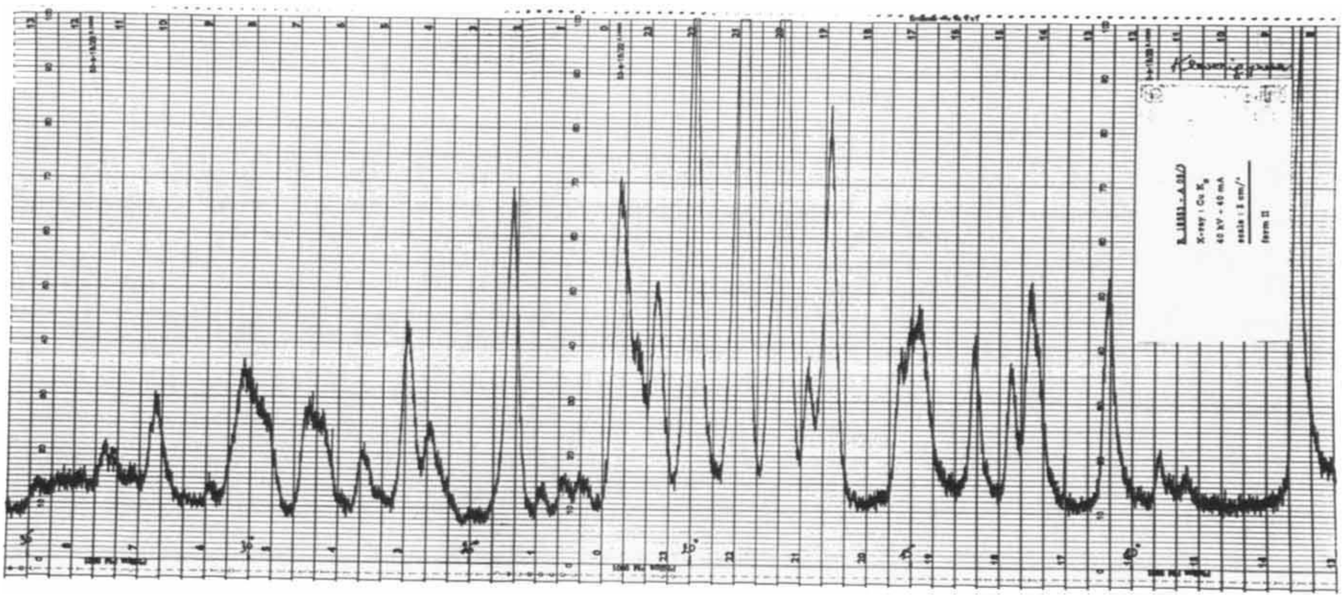


FIGURE 12. Powder X-ray diffraction pattern of Loperamide Hydrochloride Tetrahydrate

TABLE 2

Solubility of Loperamide Hydrochloride in Various Solvents

Solvent	Solubility in g/100 mL solution
water (pH = 1.7)	0.14
citrate-phosphate pH 6.1	0.008
citrate-phosphate pH 7.9	<0.001
methanol	28.6
ethanol	5.37
2-propanol	1.11
dichloromethane	35.1
acetone	0.20
ethyl acetate	0.035
diethyl ether	<0.001
hexane	<0.001
toluene	0.001
N,N-dimethylformamide	10.3
tetrahydrofuran	0.32
4-methyl-2-pentanone	0.020
propylene glycol	5.64
polyethylene glycol 400	1.40
dimethylsulfoxide	20.5
2-butanone	0.18

4. Methods of Analysis

4.1 Drug Substance

Elemental analysis for a specific lot of reference standard is given below[16]. C,H,N and O were determined on a Carlo Erba Elemental Analyzer. Chlorine was determined with a Dionex 2010 ion chromatograph.

% Calculated for $C_{29}H_{33}ClN_2O_2.HCl$		Found for Ref. Std. V860-87	Difference
C	67.83	67.60	-0.23
H	6.67	6.60	-0.07
N	5.46	5.29	-0.17
O	6.23	6.33	+0.10
Cl	13.81	13.90	+0.09

Assay of loperamide hydrochloride by non-aqueous titration of the piperidine nitrogen (one basic equivalent per mole) is described in the current USP[1]. For reference standard V860-87 a result of 99.9% was obtained[17].

The purity of loperamide hydrochloride has been determined by both thin-layer chromatograpy[1,18] (tlc) and high performance liquid chromatography[19] (HPLC). The current Janssen tlc system[18] utilizes a KC18F reversed-phase 20x20 cm precoated Whatman plate with a 1,4 dioxane/1M ammonium acetate/methanol/tetrahydrofuran (40:40:10:10 vol) solvent system. A 500 ug amount of the substance is applied as a 5% solution in dichloromethane/methanol (2:1 vol). Detection is by iodine vapors or UV at 254 nm. The limit of detection is approximately 0.2% with iodine vapors. In this system loperamide has an Rf of approximately 0.54.

The Janssen gradient elution HPLC system employs a 30 cm by 4.6 mm Hypersil ODS (5um) column. A 10 uL portion of a 1% solution in methanol is injected on the column with solvents, elution mode and instrument parameters as follows.

- elution solvents : A = 0.01 M tetrabutylammonium hydrogen sulfate in water
  B = acetonitrile
  C = methanol
- elution mode:

time (minutes)	0	20	30
% A	90	30	0
% B	10	50	80
% C	0	20	20

- flow : 2 mL per minute.
- detection method : UV at 220 nm.

In this stability indicating system loperamide hydrochloride has a retention time of approximately 13 minutes. The limit of detection is approximately 0.1ug.

4.2 Drug Product

Several systems have been developed for the analysis of loperamide hydrochloride in pharmaceutical dosage forms[20-22]. The USP method for loperamide hydrochloride capsules involves chloroform extraction followed by a colorimetric reaction with Tropaeolin 00. A normal phase HPLC employing a 25 cm by 4.6 mm 10 um silica column, using chloroform/methanol/ammonia (95.5:4.5:0.5) was reported as applicable for capsules, tablets and syrup[21]. A non-published Janssen method for capsules and tablets uses a C-18 reversed phase HPLC system with 0.5% ammonium acetate in water/acetonitrile/methanol (25:38:37) as mobile phase, a flow rate of 1.5 mL/min and UV detection at 220 nm[23].

4.3 Biological fluids

A specific and sensitive radioimmunoassay for loperamide has been developed[24]. Loperamide is well absorbed from the gastrointestinal tract, but due to a very large first pass effect, therapeutic plasma levels are extremely low[25]. The limit of detection of the assay is 0.5 ng/mL of sample.

5. Stability-Degradation

Loperamide hydrochloride is a stable drug substance at room temperature and protected from direct daylight. It is not hygoscopic[26]. The stability of the drug substance has been studied under various stress conditions[27]. The drug was stable when exposed to 17000 lux (1580 foot candles) for 7 days and in 1 N sodium hydroxide at 100°C. After 5 days in water at 100° approximately 0.1% of the loperamide hydrochloride is hydrolytically cleaved at the nitrogen of the piperidine ring. In 1 N hydrochloric acid at 100°C the drug is dehydrated forming a double bond in the piperidine ring. After 4 hours approximately 5% of the dehydrated compound is formed. With the stress conditions studied the drug is most unstable in the presence of a strong oxidant. A sample analyzed immediately after preparation of a 1.5% peroxide solution showed the presence of 3.2% of the cis N-oxide of loperamide and 2.4% of the trans N-oxide of loperamide. In all studies samples were analyzed by HPLC and decomposition compounds were identified by mass spectrometry.

The N-oxides were also identified by NMR. All structures were confirmed by synthesis of reference samples.

Loperamide hydrochloride capsules, tablets and oral liquid stability has been studied. All are stable formulations [28,29].

Plasma samples containing the drug are stable when stored frozen (-20°C)[30].

6. Drug Metabolism, Pharmacokinetics

Early work on the pharmacokinetics of loperamide in man suggested an apparent half-life of the compound in the order of 40 hours[25]. The data on which this estimate was based were collected after administration of tritium labeled loperamide. It was subsequently determined that there may have been a loss of some of the radioactivity from the molecule in-vivo resulting in the formation of tritiated water. Since the radioactivity was the parameter being monitored this phenomenon introduced an error in the estimation of the drug's apparent half-life. Subsequent studies utilizing a specific radioimmune assay procedure[24] suggested a mean half-life of 10.8 $\pm$ 0.6 hours for the half-life of this compound in man[31,32]. The half-life was comparable for a syrup formulation and a capsule dosage form. Peak plasma levels were obtained at 1.8 hours for the syrup formulation and 4.3 hours for the capsule formulation. Although the syrup formulation is more rapidly absorbed comparable maximum serum concentrations were attained for both dosage forms. The pharmacokinetic model that best fits the data was the one compartment open model with first order absorption.

Only 1% of the drug is excreted unchanged in the urine[32].

The drug is metabolized by N-demethylation of one or both methyl groups from the amide nitrogen[32].

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8. Acknowledgement

We are indebted to our colleagues at Janssen, Beerse, Belgium and Janssen, Piscataway, New Jersey for contributions to this profile.

The manuscript was expertly typed by Mrs. Audrey Rafalko.

Metipranolol

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1. Foreword

Metipranolol is a non-selective adrenergic β-blocking agent with high intrinsic sympatomimetic effect on organism. It has been first prepared in Research Institute for Pharmacy and Biochemistry /VÚFB, Prague/ *(1,2)* and pharmacologically tested there but also by many other well known world institutions.

The drug has been primarily used in the management of patients with ischemic heart disease in order to supress the acute manifestations of angina pectoris and improve the tolerance of organism against physical exertion. Favourable antiarrhythmic effect may be utilized in the medical treatment of various types of tachycardia. Additionally, metipranolol has been found effective in the treatment of hypertension and psychoses of somatic origin.

2. Description

2.1. Nomenclature

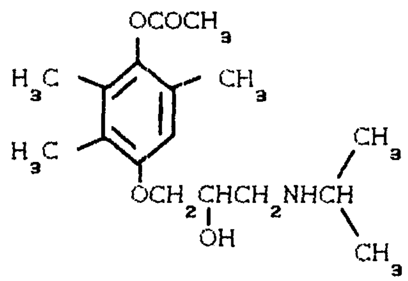
Chemical name: 1-(4-acetoxy-2,3,5-trimethylphenoxy)-3-isopropylamino-2-propanol or, alternatively, 1-(4-acetoxy-3,5,6-trimethylphenoxy)-3-isopropylamino-2-propanol.

Generic names: trimepranol, methypranol, metipranolol.

Trade names: Betamann®, Disorat®.

2.2. Formula, Molecular weight

$C_{17}H_{27}NO_4$ M_r 309.38



2.3. Elemental Analysis

	C[%]	H[%]	N[%]	O[%]
Theoretical	66.00	8.79	4.53	20.68
Experimental	66.15	8.70	4.51	–

2.4. Structural Origin

Metipranolol is a member of relatively large group of non-selective β-blockers, derived from 3-isopropylamino or 3-tert-butylamino-2-propanol, involving carazolol [63976-74- 9], alprenolol [13655-52-2], bupranolol [14556-46-8], atenolol [29122-68-7], prinodolol [13523-86-9], practolol [6673-35-4], 4-hydroxypropranolol [10476-53-6] and propranolol [525-66-6].

2.5. Used Chemical Forms

Free base [22664-55-7] is the most commonly used form for oral administration. Alternatively neutral salts of metipranolol with fumaric or tartaric acid [36592-78-6] have been recomended for parenteral usage and hydrochloride for the preparation of eye drops(*3*).

2.6. *Optical Activity*

The molecule of metipranolol contains a chiral centre (C-2) and therefore the existence of two enantiomers could be expected. Both optically active forms of metipranolol were prepared by enantioselective crystallization of the racemate with mandelic or tartaric acid and pharmacologically tested (*4*).

2.7. *Appearance, Colour, Odour*

Free base of metipranolol, fumarate and tartrate salts are white crystalline and odourless powders.

3. Physical properties

3.1. *Spectral Properties*

3.1.1. *Ultraviolet Spectrum*

The presence of aromatic ring in the molecule of metipranolol induces significant absorption in near UV region. The ultraviolet spectrum of metipranolol base in neutral methanol (1.027 mg/ml), recorded on Pye Unicam PU 8800 spectrometer, is shown in Fig.1. The spectrum exhibits two characteristic maxima at 278 nm ($A^{1\%}_{1cm}$ =51.3) and 274 nm ($A^{1\%}_{1cm}$ =50.5).

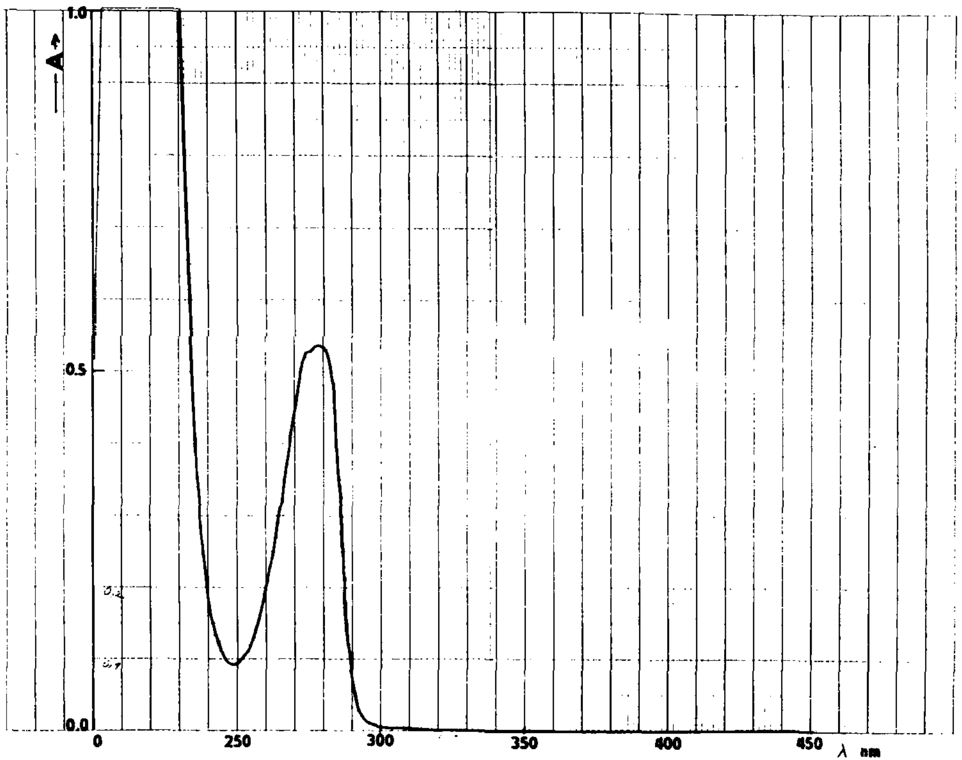


Fig.1 The ultraviolet spectrum of metipranolol base in neutral methanol.

3.1.2. Infrared Spectrum

The IR spectrum of metipranolol base in a KBr tablet (0.94 mg/200 mg KBr), recorded on Pye Unicam SP-200 G spectrometer, is shown in Fig.2. Characteristic band assignments are given in table I

Tab.I. IR spectral assignments of metipranolol base

Frequency [cm^{-1}]	Assignment
890	solit. H on Ar
1089] 1130	>CH-OH
1209] 1234	$-O-CO-CH_3$; Ar-O-R
1580] 1610	Ar
1738	$-O-CO-CH_3$
2800 - 2900	Alkyl
3040	-OH
3182	-NH-

3.1.3. Nuclear Magnetic Resonance Spectrum

The ^1H NMR spectrum of metipranolol base in $CDCl_3$ (100 mg/1.5ml) was recorded on Tesla BS 567 A spectrometer using TMS (tetramethylsilane) as an internal reference (Fig.3). Corresponding spectral assignments are summarised in table II.

The ^{13}C NMR noise decoupled and off-resonance spectrum of metipranolol base in $CDCl_3$(100mg/1.5ml; TMS-internal standard), recorded on BS 567 A spectrometer, is presented in Fig.4. The carbon chemical shift values and spectral assignments are listed in table III.

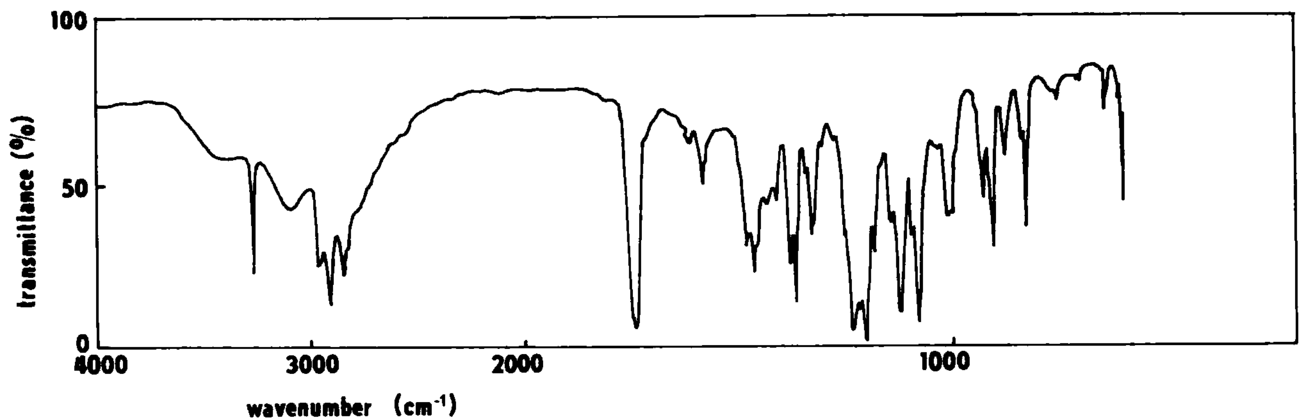
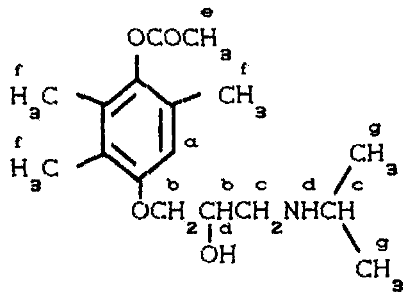


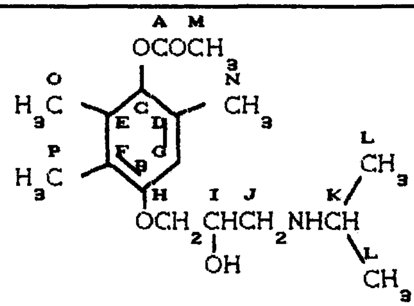
Fig.2 The IR spectrum of metipranolol base in a KBr tablet.

Tab.II. Metipranolol ^{1}H NMR assignments



Proton assignments	δ [ppm]	Multiplicity
a	6.60	broad singlet
b	3.96	multiplet
c	2.80	multiplet
d	2.36	broad singlet
e	2.34	singlet
f	2.14;2.12;2.06	singlet
g	1.09	dublet

Tab.III Metipranolol ^{13}C NMR assignments



Carbon	δ [ppm]	Carbon	δ [ppm]
A	169.48	I	68.57
B	154.17	J	49.60
C	141.92	K	48.93
D	129.75	L	23.01
E	127.28	M	20.47
F	123.99	N	16.58
G	111.37	O*	13.00
H	71.26	P*	12.03

* May be interchanged

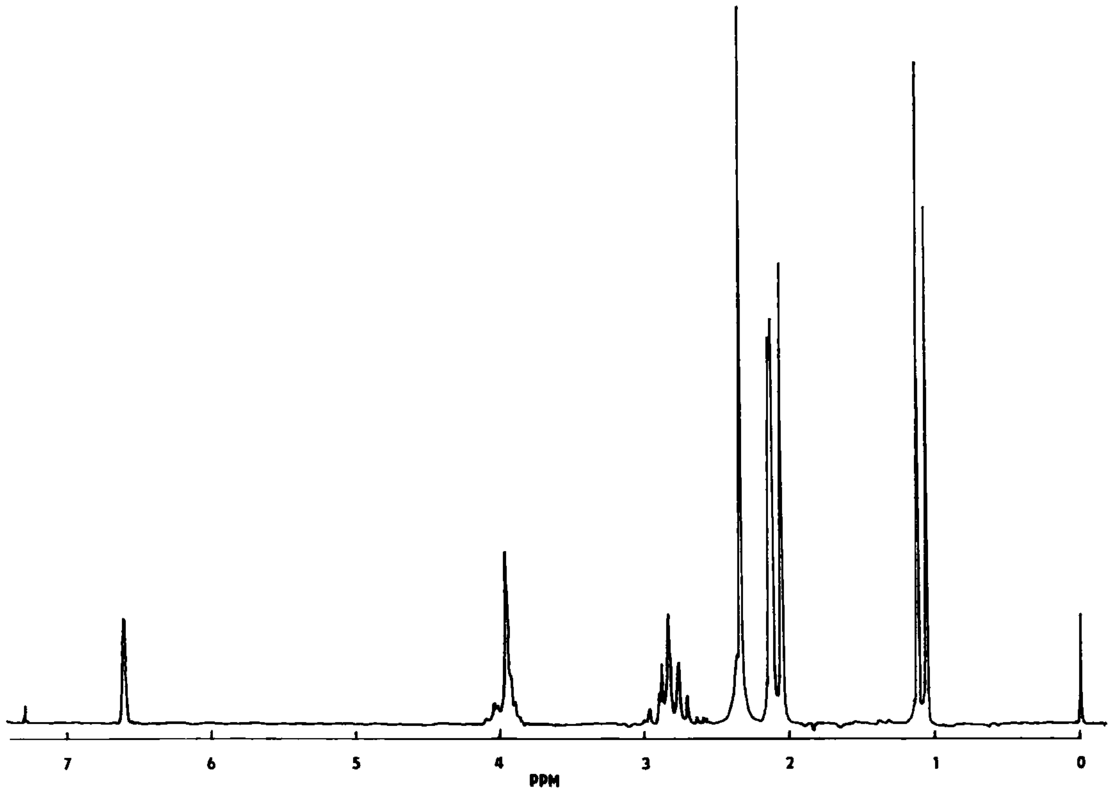


Fig.3 The ^{1}H NMR spectrum of metipranolol base in $CDCl_3$.

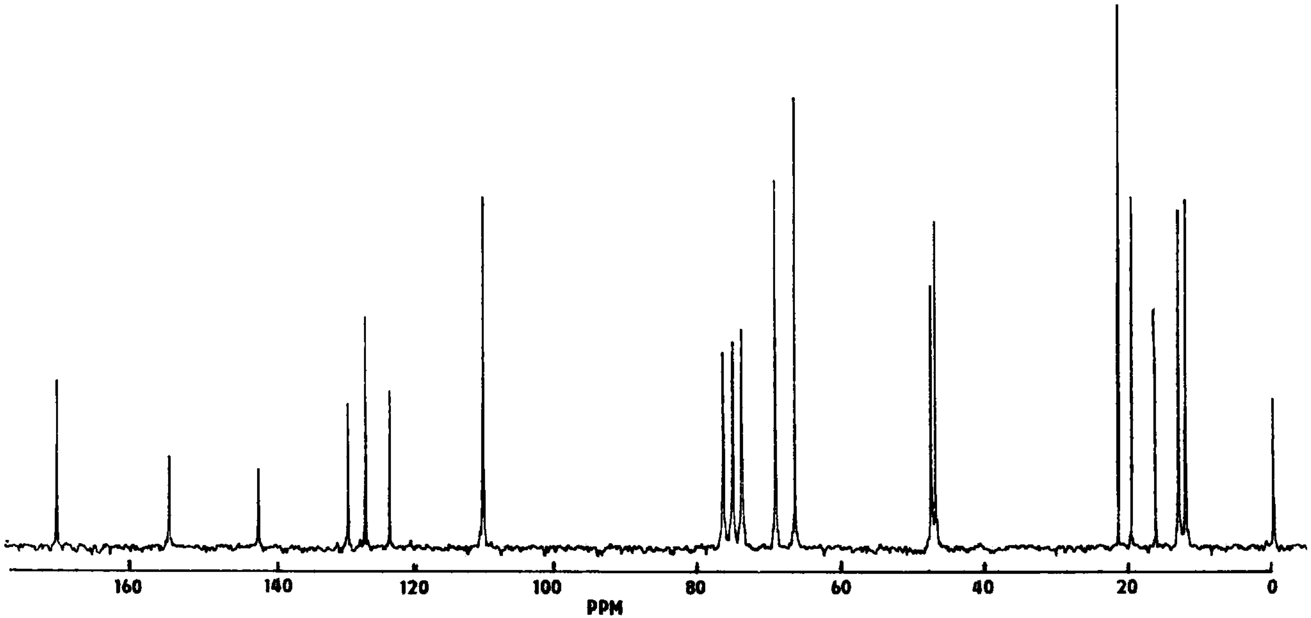


Fig.4 The ^{13}C NMR noise decoupled and off-resonance spectrum of metipranolol base in $CDCl_3$.

3.1.4. Mass Spectrum

The electron impact mass spectrum (Fig.5) was recorded on Varian MAT 44 S spectrometer with an ion source temperature 195°C, emission current 0.2 mA and electron energy of 70 eV. The list of more significant fragmentation peaks and their relative intensities is given in table IV.

Tab.IV Electron impact mass spectrum of metipranolol base. Peak intensity data.

Peak	I/Base	Mass
1	2.03%	39
2	8.14%	40
3	6.05%	41
4	3.36%	42
5	16.08%	43
6	6.36%	44
7	2.79%	55
8	8.62%	56
9	5.36%	57
10	4.07%	58
11	2.31%	60
12	1.70%	67
13	1.74%	69
14	2.18%	70
15	2.99%	71
16	100.00%	72
17	7.76%	73
18	1.98%	74
19	1.51%	79
20	1.72%	91
21	1.55%	98
22	2.37%	100
23	3.11%	102
24	3.81%	116
25	1.47%	137
26	3.40%	151
27	13.68%	152
28	1.68%	153
29	1.50%	194
30	0.13%	233
31	1.06%	265
32	0.21%	266
33	0.16%	294
34	0.29%	309

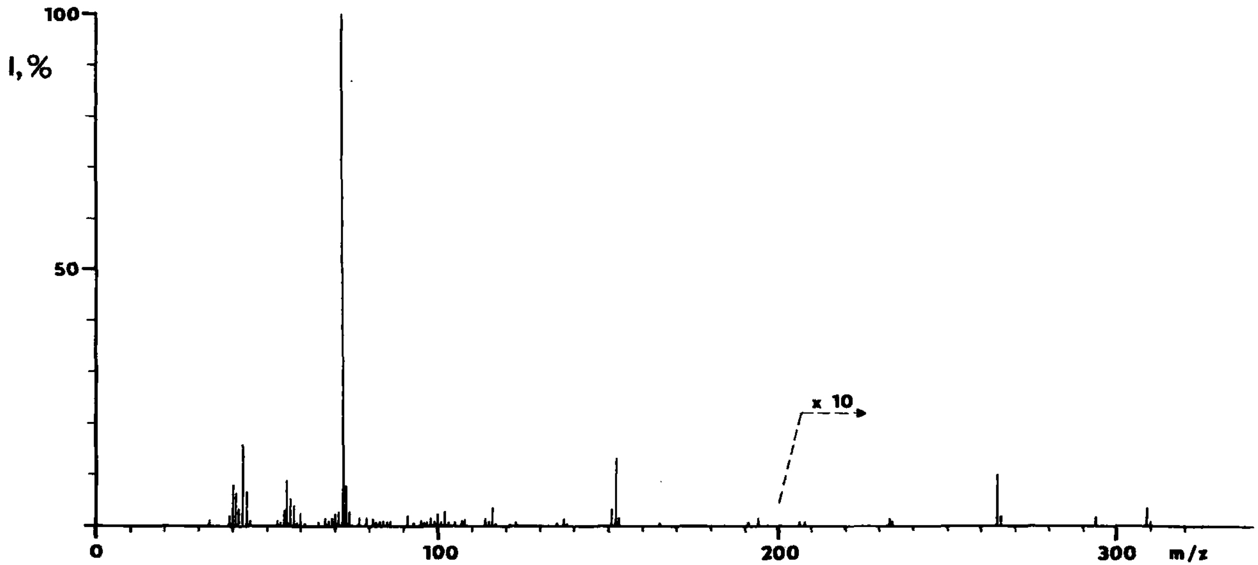


Fig.5 The electron impact mass spectrum of metipranolol base.

3.2. Thermal Properties

3.2.1. Melting Range

Metipranolol base melts between 108.5 - 110.5°C. The melting range of metipranolol fumarate is 170 - 172°C, that of neutral tartrate salt is 157 - 158°C.

3.2.2. Differential Thermal Analysis

The DSC thermogram for metipranolol base was recorded on DSC-1B differential scanning calorimeter (fig.6). The sample size was about 1 mg, the heating rate was 4°C/min. An endothermic peak occuring at about 105°C corresponds to the melting of the substance.

3.2.3. Thermogravimetry

The thermogravimetric curve for metipranolol base (fig.7) confirms that the compound is stable up to the temperature of approximately 190°C. The decrease in the sample weight above this temperature indicates the beginning of the substance decomposition.

3.3. Solubility

According to standard solubility test and nomenclature(5,6), metipranolol base should be characterized as freely soluble in 95% ethanol, chloroform and benzene, slightly soluble in ether and practically insoluble in water. The solubility of metipranolol base in water solutions could be increased substantially by inclusion complex formation with β-cyclodextrin (7). Both fumarate and tartrate neutral salts of metipranolol are freely soluble in water.

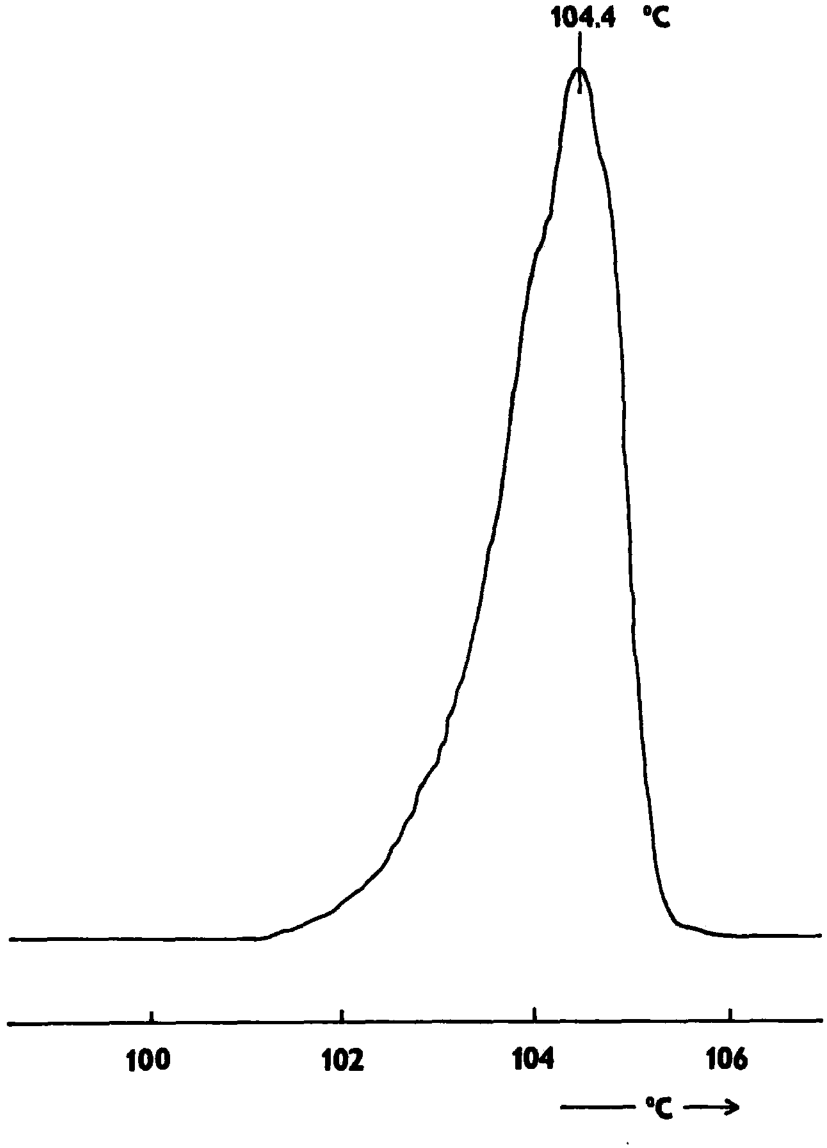


Fig.6 Differerential scanning calorimetry scan of metipranolol base.

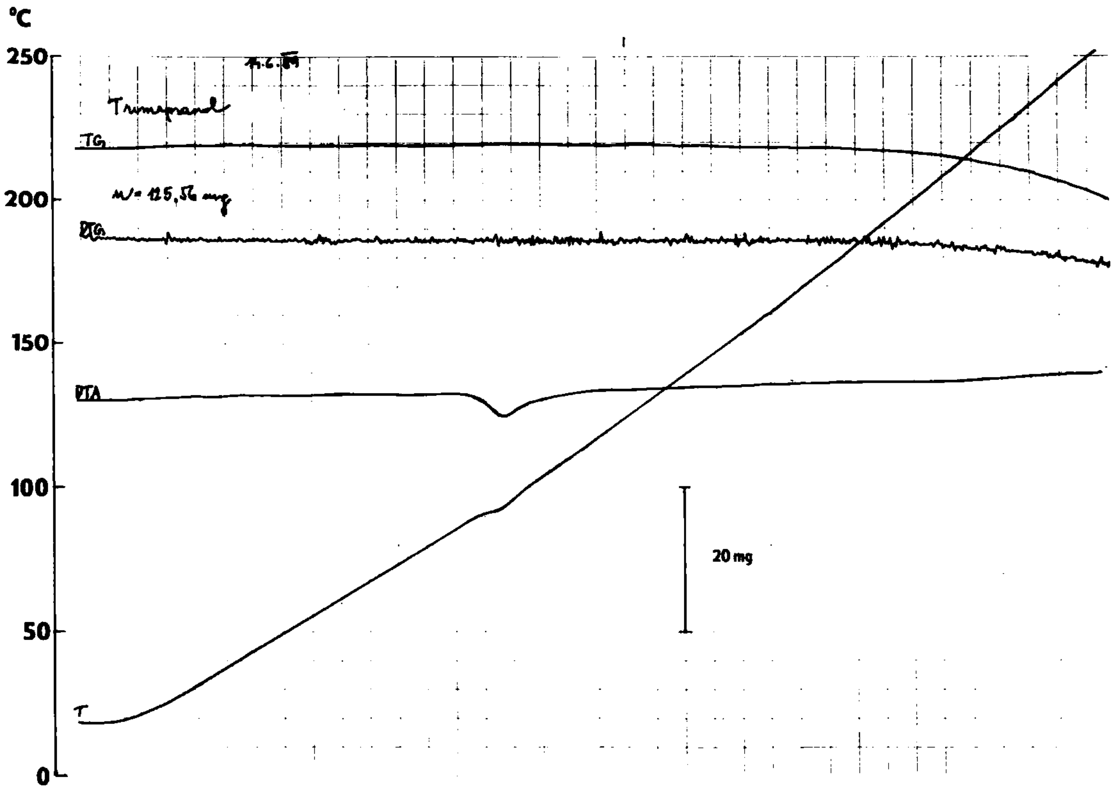


Fig.7 The thermogravimetric curve (TG) of metipranolol base

4. Synthesis

The complete reaction scheme of metipranolol base is given in fig.8. In principle it does not differ from syntheses of other biologically active aryloxyisopropanolamines. Corresponding substituted phenol (intermediate 3) is condensed with epichlorohydrine to epoxide (4) and then by reaction with isopropylamine converted to metipranolol base (5). More detailed information about single reaction steps could be found in literature *(1,2,8-10)*. Pure optical antipodes of metipranolol were isolated from racemic base by enantioselective crystallization with mandelic or tartaric acid *(4)*.

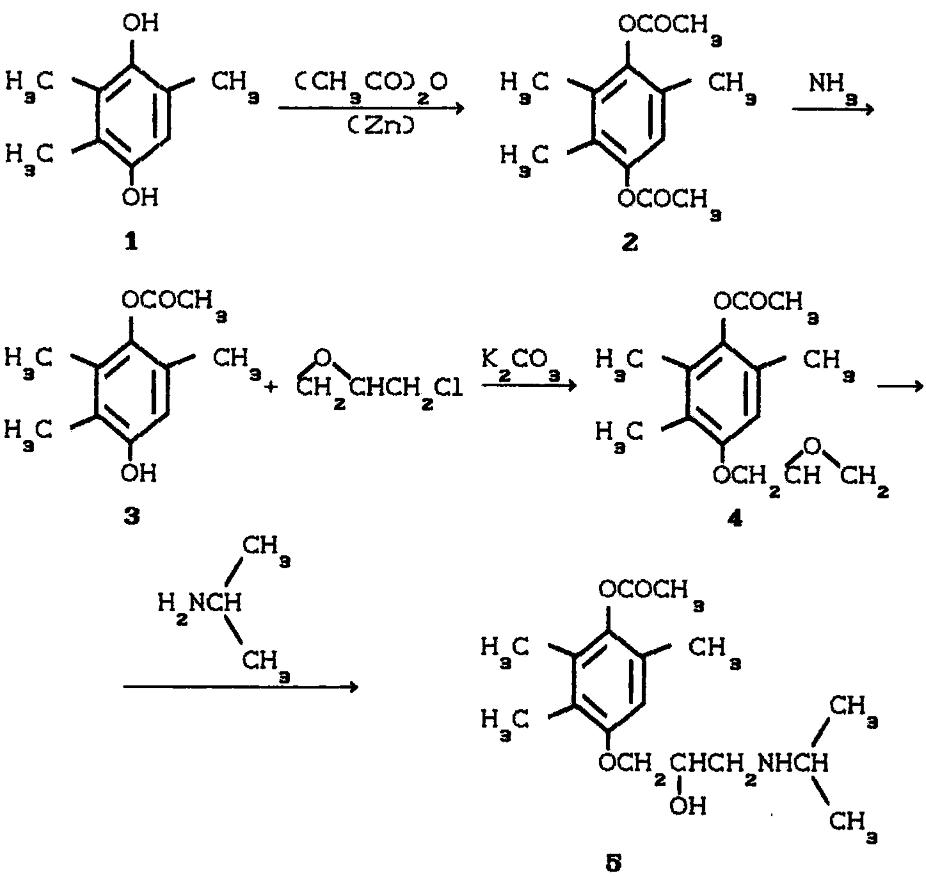


Fig.8 Reaction scheme of metipranolol base

5. Stability

From the preliminary short-term stability test *(11)* it should be noted that metipranolol quickly decomposes in alkaline (NaOH) and oxidizing (H_2O_2) solutions. Deacetylmetipranolol as a main product of alkaline hydrolysis is also primary metabolite. Metipranolol seems to be resistent against acidic hydrolysis in diluted hydrochloric and acetic acid solutions. The exposition to UV light (Hg-lamp, λ_{max} 258 nm) accelerates significantly the decomposition of metipranolol in water solution and also in substance.

Long term stability test the confirmed excellent stability of the substance and 40 mg tablets stored under various thermal conditions (5, 25, 38 and 60°C) for three years. In all regimes the sum of decomposition products (deacetylmetipranolol (DAT), trimethylbenzochinone (TMB)) did not exceed the limit declared. Less favourable is the stability of metipranolol fumarate 1% eye drops. An increase in content of decomposition products with increasing temperature and time of exposition is evident from table V.

Tab.V Stability of metipranolol fumarate 1% eye drops

Exposition [days]	Temperature [°C]	Cont.of degrad.products* DAT [mg/mL]	TMB [mg/mL]
0	25	0.059	0.002
40	25	0.085	0.006
	38	0.125	0.015
	60	0.316	0.034
90	25	0.144	0.012
	38	0.190	0.029
	60	0.400	0.050
180	25	0.130	0.019
	38	0.204	0.043
	60	0.453	0.068
365	25	0.145	0.029
	38	0.315	0.058
	60	0.908	0.086

Tab.V (continued)

Exposition [days]	Temperature [°C]	Cont.of degrad.products* DAT [mg/mL]	TMB [mg/mL]
730	25	0.193	0.032
	38	0.246	0.043
1095	25	0.207	0.058

* Determined spectrophotometrically

6. Pharmacology

Metipranolol was characterised as highly potent β-blocker with little intrinsic β-stimulating activity.The relations between some biological and physical-chemical properties in series of derivatives of metipranolol were found (*12,13*). β-adrenolytic activity decreases with increasing lipophilicity. Correlation between partition coefficients and the β-adrenolytic activity of metipranolol and its analogues was experimentally confirmed (*14*).

The effect of metipranolol on metabolic variables in patients with ischemic heart disease, hyperkinetic syndrome, hyperthyreosis and in healthy subjects after sympathetic stimulation was widely studied. Metipranolol administered orally decreases heart rate (*15,16*) and prolongs systolic time intervals (*17,18*). The antiarrhythmic effect of metipranolol in comparison with trimecaine was tested on the ventricular fibrilation threshold in anesthetized closed chest dogs. The study of bronchopulmonary and cardiovascular effects confirmed that metipranolol significantly increases the pulmonary resistence (*19*).

The ability of metipranolol to reduce blood pressure was experimentally confirmed on a group of hypertensive patients (*20*). The antihypertensive effect was more pronounced with combination of metipranolol and butizide. Metipranolol can substantially reduce post-operative ocular hypertension (*21,22*). Elevated intraocular pressure after cataract extraction was decreased by 0.6% metipranolol eye drops administered before and during the first days after surgery. The effect of

intraocular pressure decrease supporting the normalisation of intraocular liquid circulation may be successfully utilized in medical treatment of glaucoma (*23,24*).

Metipranolol and other β-blockers found their application also in neurology and psychiatry as neuroleptic agents. States of depression, anxiety, tremor and mania originated in psychosomatic disorder could be positively affected by administration of metipranolol (*25*).

Since metipranolol has been introduced into clinical practice the complex of secondary metabolic changes in exposed organism was described. Important is the influence on the activity of adenylcyclase enzyme which accelerates the formation of cyclic adenosinemonophosphate (*26,27*). Such equilibrium shift alters the metabolism of glycides and lipides. Significant decrease in glucose levels in human blood after the administration of metipranolol was observed (*28*) and explained by blocking effect on the efficacy of insulin. Increase in human plasma levels of growth hormone and secretin was found (*29*).

7. Dosage - Dosage Forms

Recomended therapeutic doses are summarised in table VI.

Tab.VI Therapeutic doses[30]

Single	Oral	0.01 - 0.04 g
	Intravenous	0.001 - 0.002 g
Daily	Oral	0.01 - 0.08 g
	Intravenous	0.001 - 0.004 g

The experimental use of various mixtures of metipranolol with other biologically active components was described. Summary and brief characterization of mixtures tested is given in table VII.

Tab.VII Metipranolol mixtures

Components	Note	Ref.
Metipranolol--Clopamide	Anti-hypertensive composition	*31*
Torrat[R] [72416-03-6] Metipranolol-Butizid	Anti-hypertensive composition	*20,32*
Metipranolol-Butizid -Dihydralazin	Combined β-blocking, diuretic and vasodilatating effect for hypertension treatment	*33*
Metipranolol-Butizid dihydralazin-(Methyl dopa-Spironolacton)	Combined treatment of hypertension	*34*
Metipranolol-Pilocarpin	Improved effect of intraocular pressure reduction	*35*
Metipranolol-Isosorbide-5-nitrate	Amplified suppression of acute angina pectoris manifestations	*36*

8. Drug Metabolism and Pharmacokinetics

Orally administered metipranolol is rapidly and almost completely absorbed. In normal volunteers, administration of metipranolol (20-40 mg) produced blood levels of 1-3 μg/mL in 20-30 min (*15,37*). The blood levels decreased with a half-life of 17 min whereas the urinary exertion proceeded with a half-life of 1-3 hours. The β-blocade due to 10 or 20 mg of metipranolol starts at 20-30 min and is extended at least up to 12 h following oral administration (*38*). Kinetic parameters depend strongly on type of pharmaceutical formulation administered. The comparative pharmacokinetic study proved statistically significant prolongation of half-life time and β-blocking effect after administration of experimental

slow-release preparation *(39)*.

Elimination is mainly by biotransformation in the liver. Metipranolol is almost completely and very rapidly metabolized to a deacetylated metabolite [57193-14-3] *(40)*. Urinary excretion of unchanged drug is approximately 4% of the dose *(41)*. As proved on normal volunteers and patients with kidney insufficiency, the renal clearance of deacetylated metabolite decreases and the terminal half-life of the metabolite increases as the degree of kidney insufficiency grows *(42)*. Maximum in blood serum concentration occurs at 1.1 h after administration.

9. Methods of Analysis

9.1. Identification

According to official compendium*(43)* two identification tests are recomended.

a) About 0.05g of metipranolol is mixed with 1mL of concentrated H_2SO_4, yellow-green coloration appears. By adding 1drop of concentrated HNO_3 intensive brown to red coloration appears. By diluting with 5.0 mL H_2O the solution turns yellow and precipitates.

b) About 0.1g of metipranolol mixed with 5mL of diluted NaOH solution is warmed in water bath for 15 minutes. Then the solution is diluted with 5mL of water, cooled and acidified with diluted H_2SO_4. By adding two drops of fresh $K_3[Fe(CN)_6]$ solution and two drops of $FeCl_3$ solution, intensive blue coloration appears.

9.2. Spectrophotometry

Well defined absorption maximum seems to be

convenient for direct spectrophotometric assay of metipranolol in substance and various pharmaceutical formulations. Nevertheless the choice of optimal wavelength ensuring maximum selectivity and high sensitivity may be critical due to possible peak coincidentions of metipranolol, its impurities and degradation products or other absorbing components from placebo.

Metipranolol has been determined spectrophotometrically in methanol (λ_{max}=297.5nm,concentration range 20-320 μg/mL) 0.1M H_2SO_4 in water (λ_{max}=277nm, concentration range 20-400 μg/mL) and 0.1M NaOH in water-methanolic (1+9) solution (λ_{max}=238nm, concentration range 5-85 μg/mL or λ_{max}=296nm, concentration range 15-240 μg/ml) *(44,45)*. Spectrophotometric determination based on the quantification of quinone as a product of oxidation of metipranolol with $KBrO_3$ or $Ce(SO_4)_2$ *(46)* made it possible to determine low concentrations of metipranolol and deacetylmetipranolol in biological material. An introduction of suitable pre-extraction step together with alcaline hydrolysis prior to the measurement (formation of deacetylderivative) makes it possible to determine accurately the levels of metipranolol in the mixture with chlorthalidon and DH-ergocrystin*(47)*.

9.3. Chromatographic Methods

Sensitive and selective chromatographic techniques were used predominantly for analytical evaluation of complicated pharmaceutical formulations without sample pre-treatment and for monitoring of drug serum levels for the purposes of pharmacokinetic studies.

9.3.1. Paper and Thin Layer

Both techniques were utilized for identification and semiquantitative estimation of

metipranolol and its deacetylderivative in substance and drug formulations *(48)*. Commonly used conditions for separation are given in table VIII. Microgram quantities of metipranolol were successfuly determined through TLC separation and fluorimetric detection of dansylderivative *(49)*.

Tab.VIII Conditions for the separation of metipranolol

	Paper chromatogr.	TLC
Sample preparat.	1% ethanolic sol.	2% ethanolic sol.
stationary phase	Whatman 4 impregnated with 40% EtOH solution of formamide and 5% ammonium formate	Kieselgel GF/254/ WOELM-2 plates
mobile phase	chloroform	Chloroform-methanol-acetone-acetic acid-water(50:10:20:10:5)
detection	1)UV_{254} quenching 2)Spraying with 8% phosphomolybdenic acid in EtOH 3)Spraying with Dragendorff agent	1)UV_{254} quenching 2)Spraying with acidic potassium permanganate solution 3)Spraying with $FeCl_3$+$K_3[Fe(CN)_6]$ water solution 4)Densitometry *(47)*

9.3.2. High Performance Liquid

A sensitive,selective and reproducible assay for simultaneous determination of metipranolol and its active metabolite α-hydroxymetipranolol in plasma was described *(50)*.The whole analytical procedure consists of extraction step and HPLC analysis on 5μm silica B/5 stationary phase using hexane-isopropanol-methanol-concentrated ammonium

hydroxide (850:100:50:1) mixture as mobile phase. When analysed on reversed phase HPLC columns it is recomended to suppress positive charge of the molecule of metipranolol in order to obtain sharp, non-difussion peak. Ion pair HPLC on reversed phase with 1-heptanesulfonic acid as a counterion proved to be suitable for the resolution and quantitative evaluation of metipranolol and other structurally related β-blockers in the mixture (*51*). Complete resolution of metipranolol and its deacetyl-derivative could be achieved on μBondapak C_{18} column using acetonitrile-water (1:1) mobile phase with added 1-hexanesulfonic acid as a counterion (*52*). Simple simultaneous determination of propranolol, metipranolol and atenolol on LiChrosorb C_{18} 10μm stationary phase using phosphate buffer with octyl sodium sulfate as mobile phase has been described (*53*).

Separation of metipranolol enantiomers after preparation of diastereomeric derivatives with symmetrical anhydrides of Boc-L-Ala and Boc-L-Leu was described (*54*). Proposed experimental conditions (stationary phase-μBondapak C_{18}; mobile phase-phosphate buffer pH 3.0 with the addition of acetonitrile) made it possible to monitor serum levels of both enantiomers for pharmacokinetic purposes.

9.3.3. Gas Chromatography

Gas chromatography was used for monitoring of drug levels in various biological fluids. Simple determination of underivatized metipranolol and propranolol in urine after extraction with organic solvent (petroleum ether - 2-methylbutanol; 7:3) has been reported (*55*).

The use of sensitive EC detector requires suitable derivatization step prior to GC. Metipranolol has been converted to electron-capturing derivatives with methyldichlorophosphine and sulfur (*56*) or 2,4-dichlorobenzeneboronic acid (3,5-bis(trifluoromethyl)benzeneboronic acid) (*57*). In another procedure metipranolol was transferred to perfluoroacylderivatives with trifluoroacetic

anhydride or N-heptafluorobutyrylimidazole *(58,59)*. Retention indices of metipranolol, some other β-adrenolytics and their perfluoroacylderivatives were published *(60)*.

Direct enantiomeric resolution of metipranolol racemate derivatised with phosgene to corresponding 2-oxazolidone was achieved on chiral polysiloxane XE-60-l-valine-(R)-α-phenylethylamide stationary phase *(61)*.

9.4. Titration - Electrochemical Determination

Metipranolol may be assayed in acetic acid containing KBr and HCl by titration with 0.1N $KBrO_3$. The endpoint is determined biamperometrically using double Pt electrode *(48)*. This method proved to be sufficiently selective and suitable for the determination of metipranolol in substance, various pharmaceutical formulations and also in biological material*(46)*.

Direct voltametric determination of metipranolol (I) and deacetylmetipranolol (II) based on the anodic oxidation on a graphite electrode has been described *(62)*. Determination of I can be done in a concentration range 9.10^{-3} to 1.10^{-4} mol/L and of II in a range 1.10^{-3} to $1.5.10^{-5}$ mol/L.

9.5. Determination of Trimepranol and its Metabolites in Biological fluids

In addition to cited and briefly described standard GC and HPLC techniques, gas chromatography-mass spectrometry (GC-MS) combination has been utilised for monitoring and identification of metipranolol and its metabolites in biological fluids. A typical analytical procedure consists of extraction and derivatization step (obviously trifluoroacetylation) followed by GC separation and on-line detection using quadrupole mass spectrometer.

Due to the high biological background it is

advantageous to use chemical ionisation mass spectrometric method producing high-mass molecular ions of greater relative intensities than electron--impact mass spectrometry. Highly specific and sensitive measurement of deacetylmetipranolol in plasma was achieved with methane used both as the carrier gas and chemical ionization reactant *(63,41)*. Metipranolol and other β-blockers, together with their metabolites, were differentiated and identified in urine by computerized GC-MS *(64)*. Retention indices (OV-101 stationary phase) and reference mass spectra were documented.

Acknowledgements

The authors would like to thank Mrs. M. Hajkova, ing.J. Holubek, ing.I.Koruna, Dr.M. Peterkova, Dr.M.Ryska, and Dr.Z.Volkova (VUFB, Prague) for kindly provided spectral and thermal data and helpfull assistance in their interpretation.

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NIZATIDINE

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TABLE OF CONTENTS

1. DESCRIPTION

1.1 Name, Formula and Molecular Weight

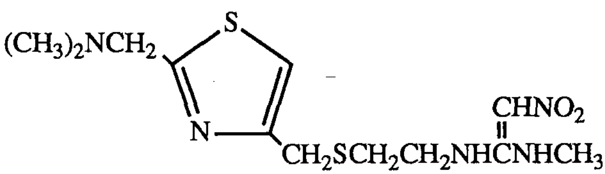
Nizatidine is marketed by Eli Lilly and Company under the trade name, Axid®. It was referred to as LY139037 in the early literature.

Chemically it is known as N-[2-[[[2-[(dimethylamino)methyl]-4 thiazolyl] methyl] thio] ethyl] -N'-methyl-2-nitro-1,1-ethenediamine. The CAS Registry Number is 76963-41-2.

Empirical Formula: $C_{12}H_{21}N_5O_2S_2$

Molecular Weight: 331.46

Structure:



1.2 Appearance, Color and Odor

Nizatidine is an off-white to buff crystalline solid. A slight sulfur-mercaptan odor may be present.

1.3 History

Nizatidine is a specific, potent H_2-receptor antagonist (1-2). Unlike cimetidine, which contains an imidazole ring structure, or ranitidine, which contains a furan ring structure, nizatidine has a thiazolyl ring structure. This structure is more potent than cimetidine in inhibition of gastric acid secretion induced by various stimuli and lacks cimetidines's anti-androgenic and hepatic microsomal enzyme inhibiting effects (3-6). The drug substance has been used in the treatment of duodenal ulceration. Nizatidine is administered as a 150 mg or 300 mg capsule.

2. SYNTHESIS

The cyclization of dimethyaminothioacetamide (I) with ethyl bromopyruvate (II) in refluxing ethanol gives ethyl 2-(dimethylaminomethyl)-4-thiazolecarboxylate (III), which is reduced with lithium triethylborohydride in tetrahydrofuran yielding 2-(dimethylaminomethyl)-4-thiazolemethanol (IV). The condensation of (IV) with 2-aminoethanethiol (V) by means of 48 percent hydrogen bromide affords 2-(dimethylaminomethyl)-4-(2-aminoethylthiomethyl) thiazole (VI), which is finally condensed with 1-(methylthio)-2-nitro-N-methylethyleneamine (VII) in water (7). The synthesis is illustrated in Figure 1.

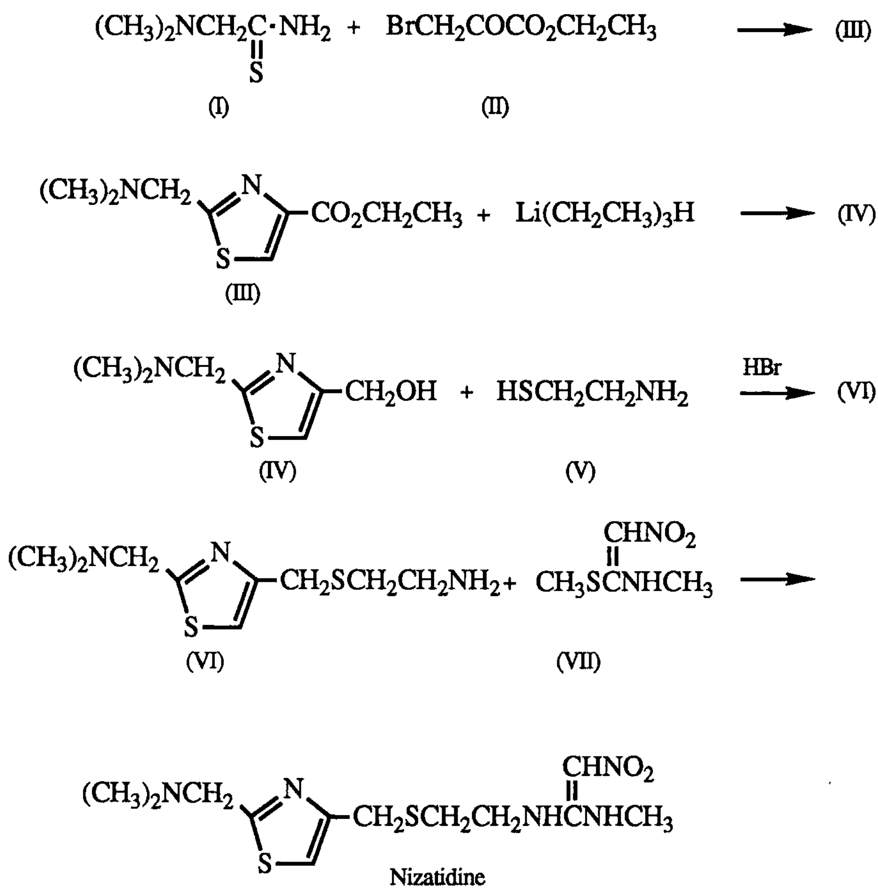


Figure 1. Chemical synthesis for nizatidine.

3. PHYSICAL PROPERTIES

3.1 Infrared Spectrum

The infrared spectrum for nizatidine as a potassium bromide pellet is illustrated in Figure 2. The spectrum was recorded on a Nicolet Model 5SXC Fourier Transform infrared spectrophotometer. The major adsorption bands for the infrared frequencies and the corresponding assignments are listed in Table I.

The infrared spectra of nizatidine in a variety of solvents at room temperature indicate the presence of two species in solution (8-9). These species differ most in the region of the nitroketaminal group and have been confirmed to be cis/trans isomers around the double bond. This is evidenced by the presence of both intramolecularly non-bonded and H-bonded N-H structure in nonprotic solvents (8).The hydrogen-bonding observed in the infrared spectra was found to involve the nitro group. The absence of a C=C stretch provides additional information regarding the delocalization of the electrons in the nitroketaminal system. Such delocalization is consistent with the ultraviolet and NMR spectral data, indicating extensive delocalization of the amine nitrogen lone-pairs throughout the system.

Table I. Infrared Band Assignments for Nizatidine

Wavenumber, cm^{-1}	Infrared Assignment
3280, 3210	NH stretch; two groups
3107	CH stretch in NO_2 - CH -
3094	CH stretch in thiazole ring
2945, 2860 2829, 2784	CH stretches in NCH_3, CH_2CH_2
1622	C=C, conjugated with NO_2
1587	Asym. NO_2 stretch, conjugated with C-C; thiazole ring, weak contribution
1521	Thiazole ring
1470, 1458 1435,1422	CH deformation in NCH_3, CH_2; CN stretch
1377, 1359	Thiazole ring for one frequency is sym NO_2, H-bonded, conjugated.

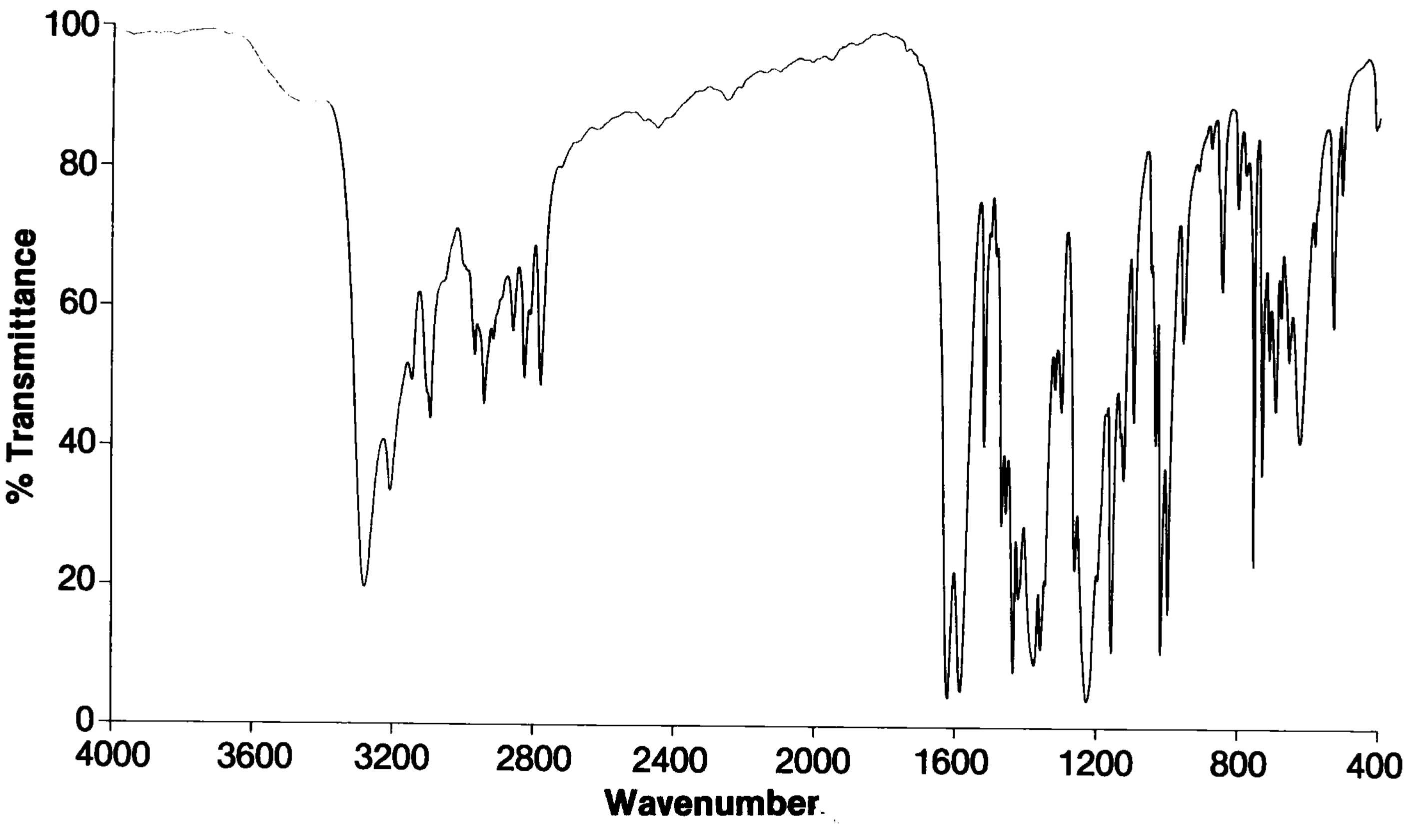


Figure 2. Fourier transform infrared spectrum of nizatidine.

3.2 Nuclear Magnetic Resonance

The 300 MHz ^1H spectrum of nizatidine (100mg/mL) in $CDCl_3$ with 0.5% v/v tetramethylsilane is shown in Figure 3. The spectrum was obtained on a Varian Unity spectrometer using the following instrumental parameters: 5 mm $^1H/^{13}C$ dual probe; spectral width, 4811 Hz; 50° pulse width; 64K time-domain data points; acquisition time, 6.8 seconds; 100 scans and probe temperature, 27°C.

The proton-decoupled ^{13}C spectrum of nizatidine (100 mg/mL) in $CDCl_3$ with 0.5% v/v tetramethylsilane is shown in Figure 4. The data were obtained using a 5 mm $^1H/^{13}C$ dual probe; spectral width, 20 KHz; 90° pulse width; 64K time-domain data points; acquisition time, 1.6 seconds; relaxation decay, 2.4 seconds; WALTZ-16 proton decoupling; 4000 scans and probe temperature, 27°C. The spectrum was processed with 1.0 Hz Lorentzian line broadening followed by the addition of 64K zero-fill data points.

Nizatidine exists as a mixture of cis/trans isomers of the nitroketaminal moiety in solution (Figure 5). The presence of a doublet resonance at approximately 95-100 ppm in the ^{13}C spectrum eliminates several possible tautomers. This is further supported by the occurrence of peak doubling of the N-methyl and N-methylene resonances of the nitroketaminal region in both the ^1H and ^{13}C spectra. The group can exist in either of two conformations of similar energy, and these interconvert slowly enough to lead to doubling of many of the NMR resonances. This is evidenced by NMR and infrared studies of nizatidine at different temperatures in solution (8-9). The two hydrogen-bonded NH groups come into resonance near 10 ppm, while the nonhydrogen-bonded NH groups come into resonance in the 6-7 ppm region of the ^1H spectrum.

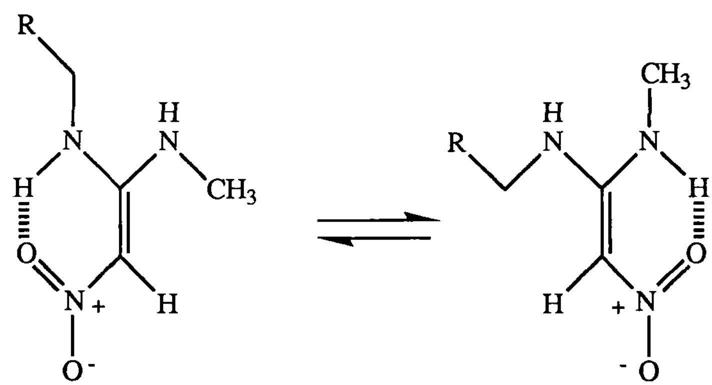


Figure 5. Cis/trans isomerization of nizatidine in the nitroketaminal region

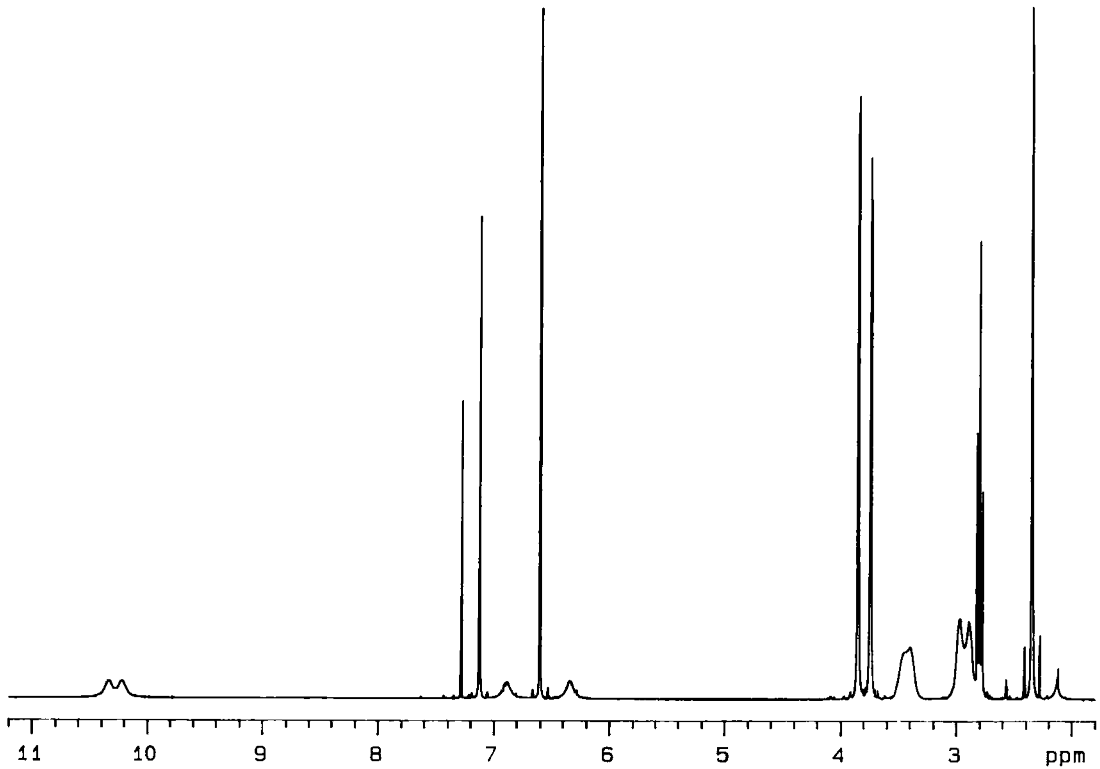


Figure 3. ^{1}*H NMR spectrum of nizatidine in* $CDCl_3$ *(27°C).*

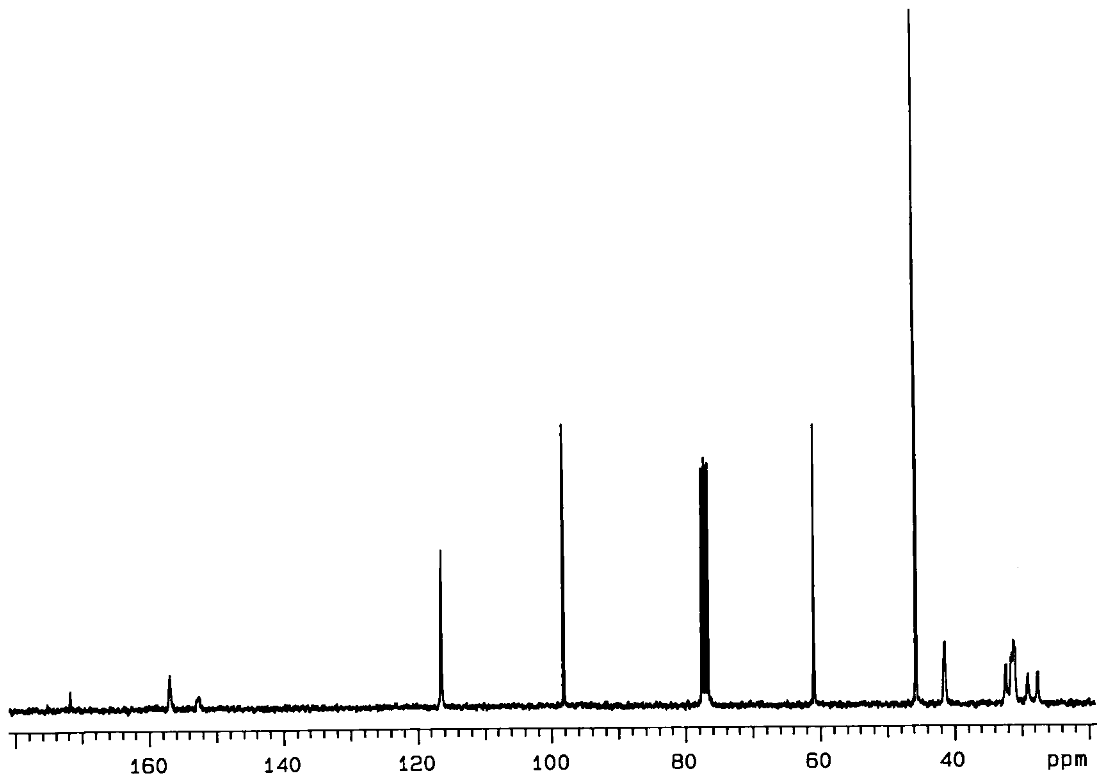


Figure 4. ^{13}C NMR spectrum of nizatidine in $CDCl_3$ (27°C).

The ^1H and proton-decoupled ^{13}C NMR spectra of nizatidine at probe temperatures ranging from -3°C to 57°C (100mg/mL in $CDCl_3$) are shown in Figures 6 and 7, respectively. The ^1H spectrum of nizatidine at 57°C shows one set of signals due to fast interconversion of the rotamers (Figure 6). When the probe temperature is lowered to -3°C, the interconversion is much slower and two sets of signals are observed for all the protons associated with the dialkylaminonitroethene group. Spin decoupling experiments on the two NH quartets and two NH triplets confirmed the assignments of the signals for the individual rotamers.

Structural assignments for both proton and carbon nuclear magnetic resonance spectra are listed in Table III.

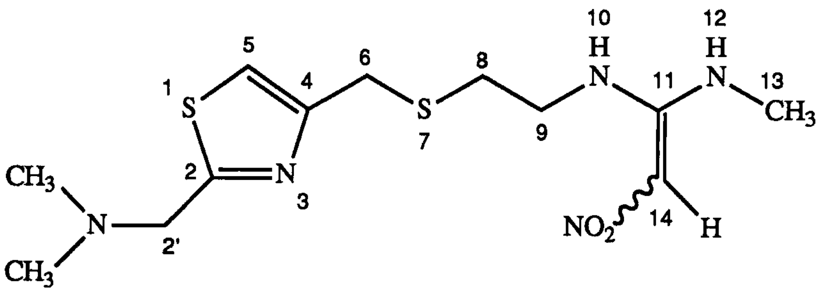


Table III. NMR Chemical Shift Assignments of Nizatidine at 3°C

Site	^1H	^{13}C
2		171.77, 172.06
4		152.28, 152.72
5	7.15	116.45, 116.59
2'	3.74, 3.77	60.77, 60.87
$N(CH_3)_2$	2.36	45.73, 45.76
6	3.85, 3.86	30.98, 32.22
8	2.79	30.65, 31.24
9	3.40, 3.49	41.04, 41.51
10	6.72, 10.35	
11		156.52, 156.74
12	7.25, 10.24	
13	2.84, 3.00	27.73, 29.07
14	6.63, 6.64	98.21, 98.26

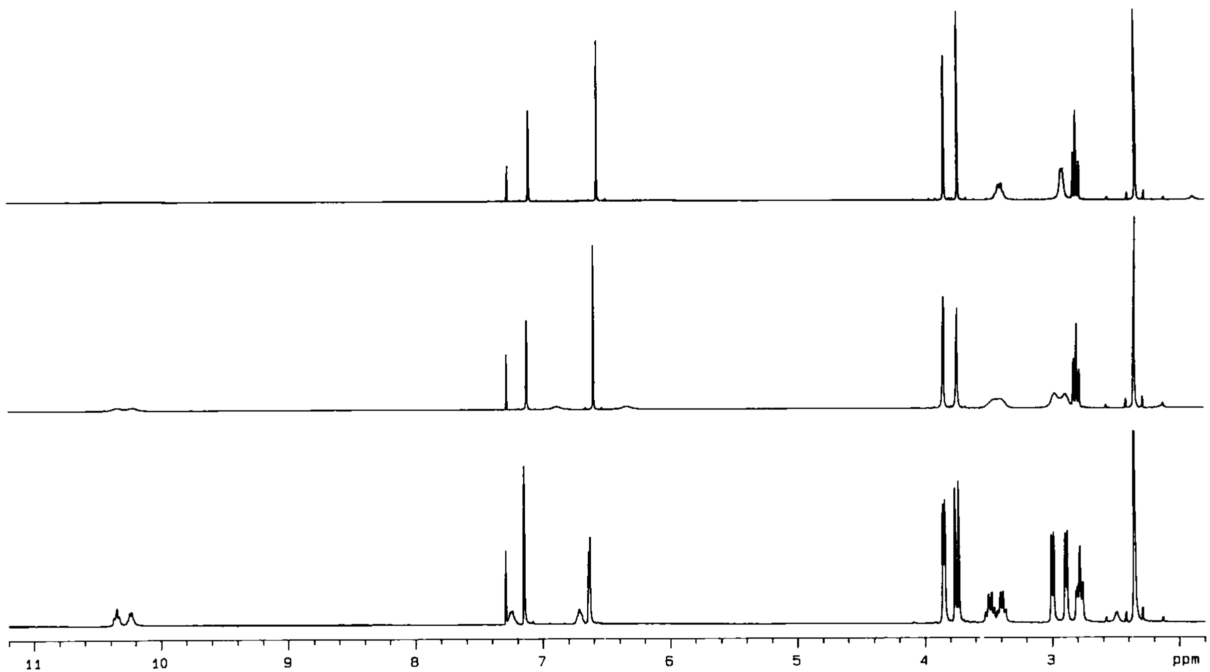


Figure 6. [1]*H NMR spectrum of nizatidine in* $CDCl_3$ *at -3°C (bottom), 27°C (middle) and 57°C (top).*

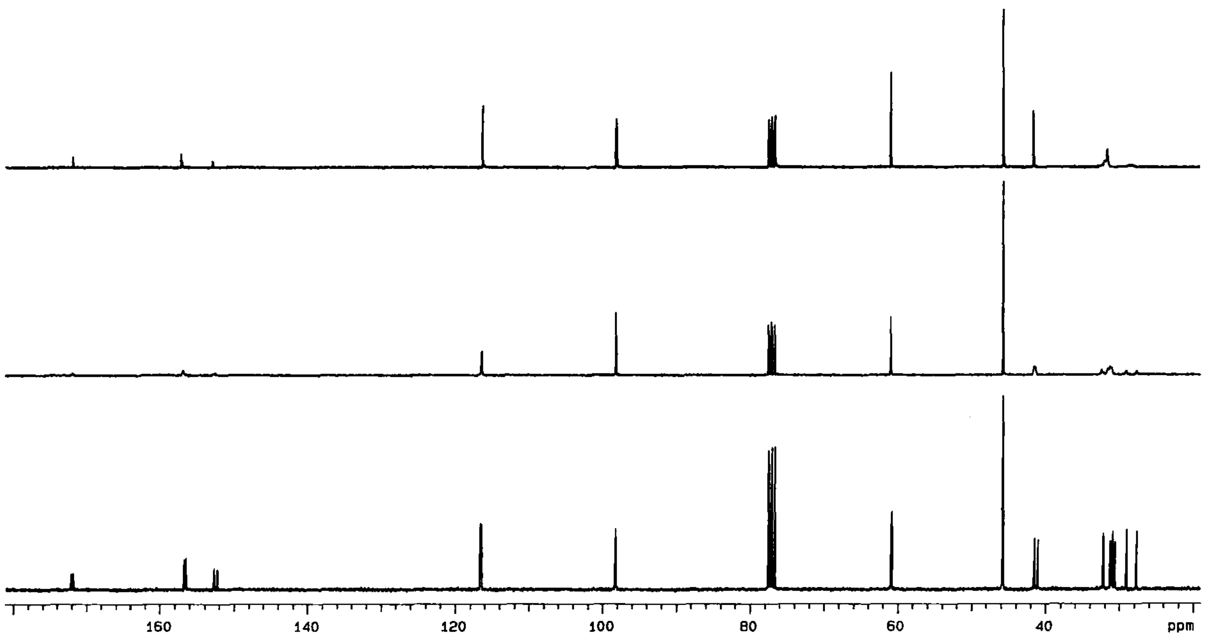


Figure 7. ^{13}C NMR spectrum of nizatidine in $CDCl_3$ at -3°C (bottom), 27°C (middle) and 57°C (top).

3.3 Mass Spectrum

The electron impact mass spectrum of nizatidine is shown in Figure 8. The spectrum was obtained using a VG Model 7070E magnet sector instrument. No molecular ion was observed in the electron impact spectrum of nizatidine. Loss of a hydroxl radical from the molecular ion of nizatidine to yield an ion at m/z 314 is consistent with other compounds having the nitroketaminal group. The complexity of the rest of the EI spectrum was resolved by determining the accurate mass of the fragment ions to better than ± 0.005 mass units. This allowed for the determination of the elemental compositions of each fragment. The ion at m/z 58 could have been $(CH_3)_2NCH_2$, C_2H_2S or CNO_2 which have masses of 58.0657, 57.9877 and 57.9929, respectively. An accurate mass measurement of the m/z 58 fragment showed $(CH_3)_2NCH_2$ to be the correct assignment. Several of these fragment assignments are illustrated in Table IV and Figure 9.

Table IV. Mass Assignments for Electron Impact Spectrum

Mass Assignment	Formula
314.110	$C_{12}H_{20}N_5OS_2$
297.102	$C_{12}H_{19}N_5S_2$
254.068	$C_{10}H_{14}N_4S_2$
227.054	$C_9H_{13}N_3S_2$
171.009	$C_6H_7N_2S_2$
111.015	C_5H_5NS
58.063	C_3H_8N

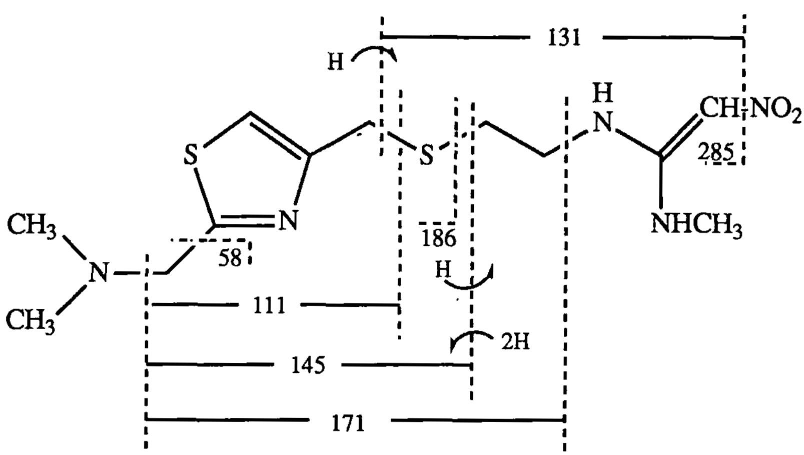


Figure 9. Electron impact fragmentation scheme for nizatidine.

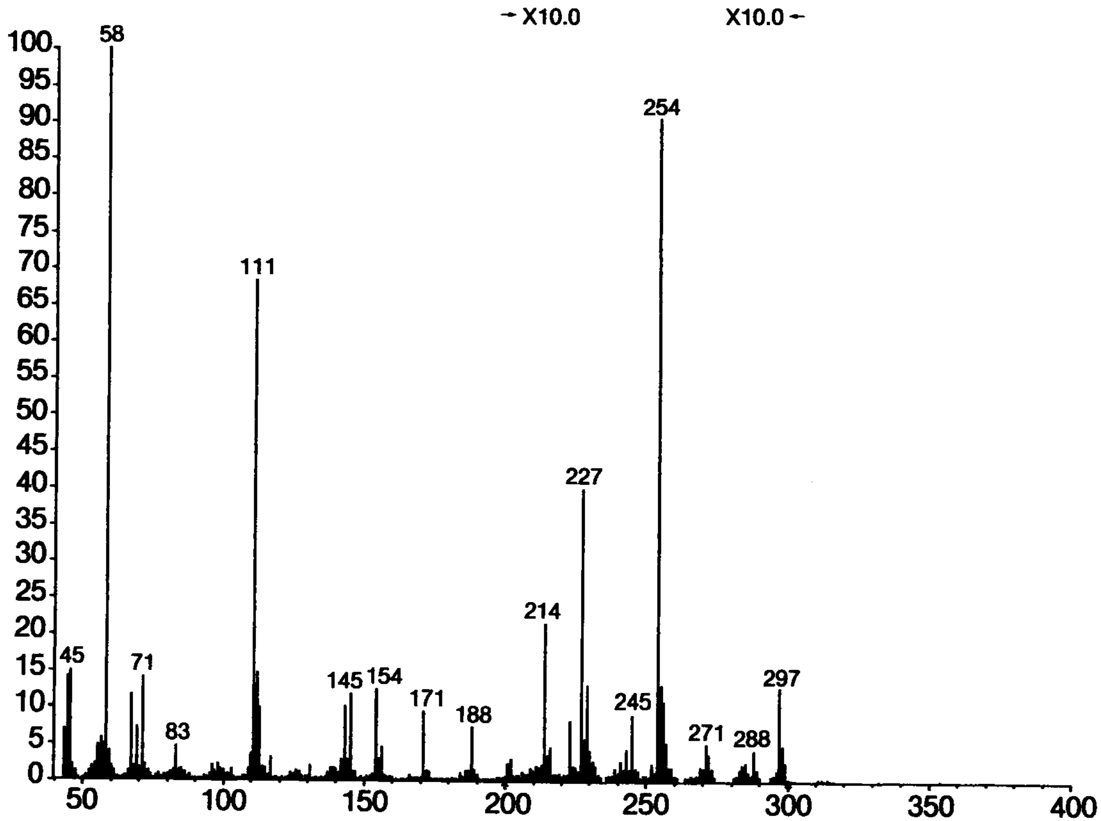


Figure 8. Electron impact mass spectrum of nizatidine.

The field desorption mass spectrum of nizatidine is shown in Figure 10. The spectrum was obtained using a Varian-MAT Model 731 double sector instrument. The spectrum consists of the protonated molecular ion at m/z 332 and a dimer ion at m/z 663.

3.4 Ultraviolet Spectrum

The ultraviolet spectra of nizatidine in methanol and water are shown in Figure 11. Spectral acquisition was performed on a Perkin Elmer Model Lambda 6 spectrophotometer using 1-cm quartz cells. The ultraviolet spectra in both methanol and water contain two absorption maxima, both of which are abolished by the addition of acid and restored by the addition of base. The maximum absorbance from the diaminonitroalkene chromophore occurs at 325 nm (methanol) and 314 nm (water) with molecular absorptivities of e=19,600 and e=15790, respectively. The peak absorbances for methanol and water solutions are listed in Table V.

The pH dependence of the UV spectrum is critical for nizatidine. At pH values below 4.5, a hypochromic effect is observed for the chromophore assignable to the substituted conjugated diaminonitroalkene. The lack of chromophore at low pH, attributable to loss of conjugation by protonation of the nitro group-bearing carbon atom in aqueous solution and which is complete in hydrochloric acid, is shown in Figure 12.

Table V. UV Absorbances and Molecular Absorptivities

Methanol			Water		
λ	E 1%/1cm	e	λ	E 1%/1cm	e
240	258	8400	260	357	11820
325	592	19600	314	476	15790

3.5 Melting Range

Nizatidine melts between 132-133°C using the USP XXII procedure for Class 1 substances.

3.6 Differential Thermal Analysis

The DTA thermogram for nizatidine, at a heating rate of 5°C per minute, shows a sharp endotherm at 133°C indicating a melt. A large exotherm occurs at 180°C where the compound begins to decompose.

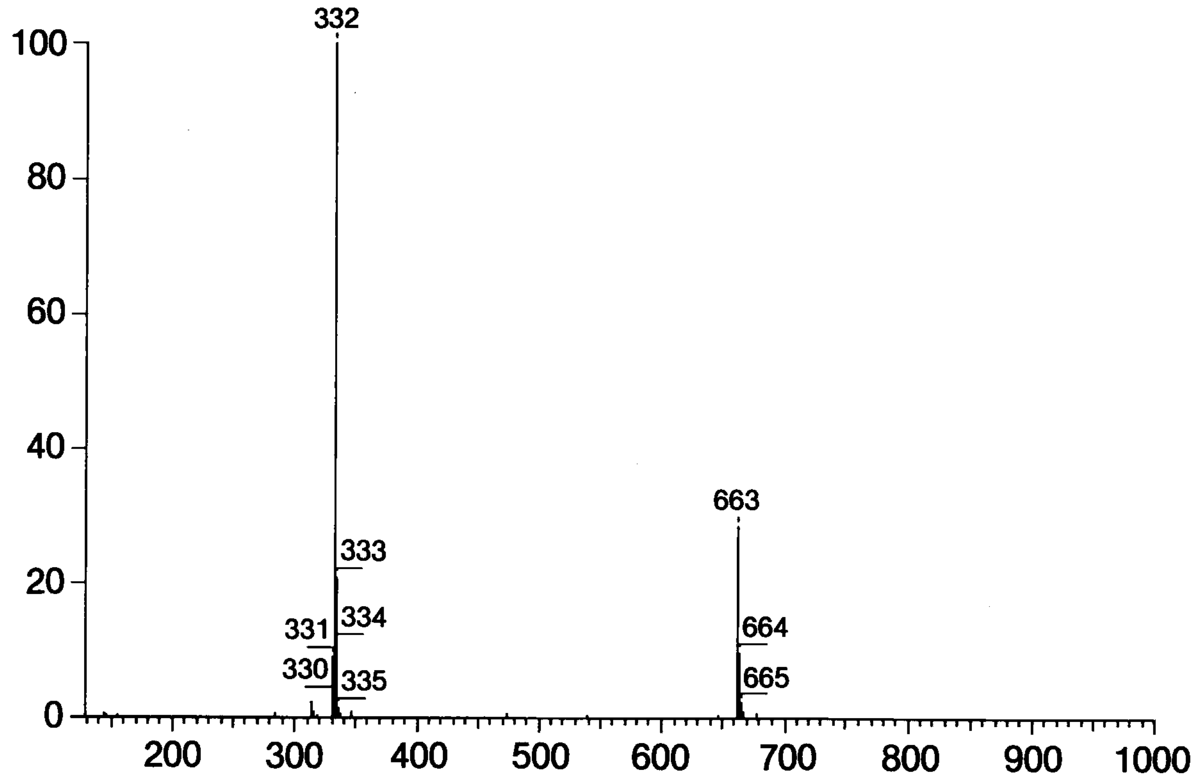


Figure 10. Field desorption mass spectrum of nizatidine.

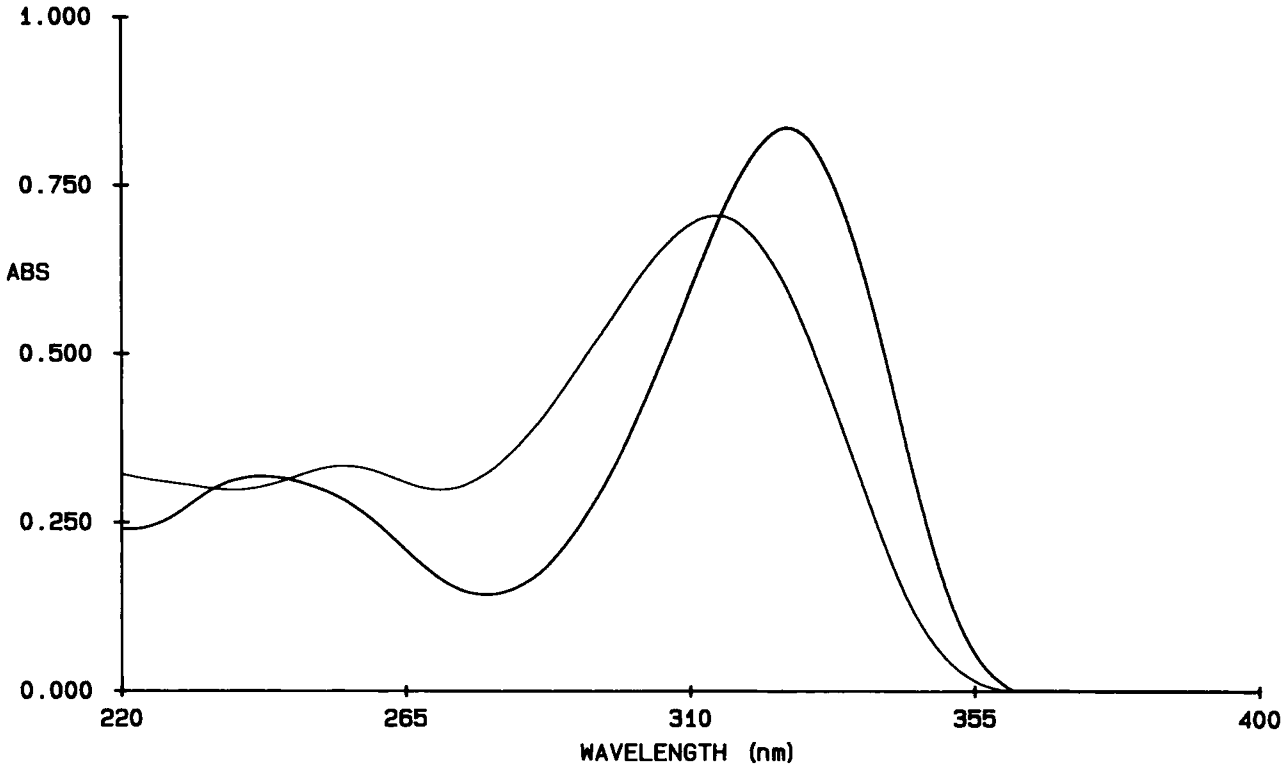


Figure 11. Ultraviolet absorption spectra of nizatidine, in water (λ_{max} = 314 nm) and methanol (λ_{max} = 325 nm).

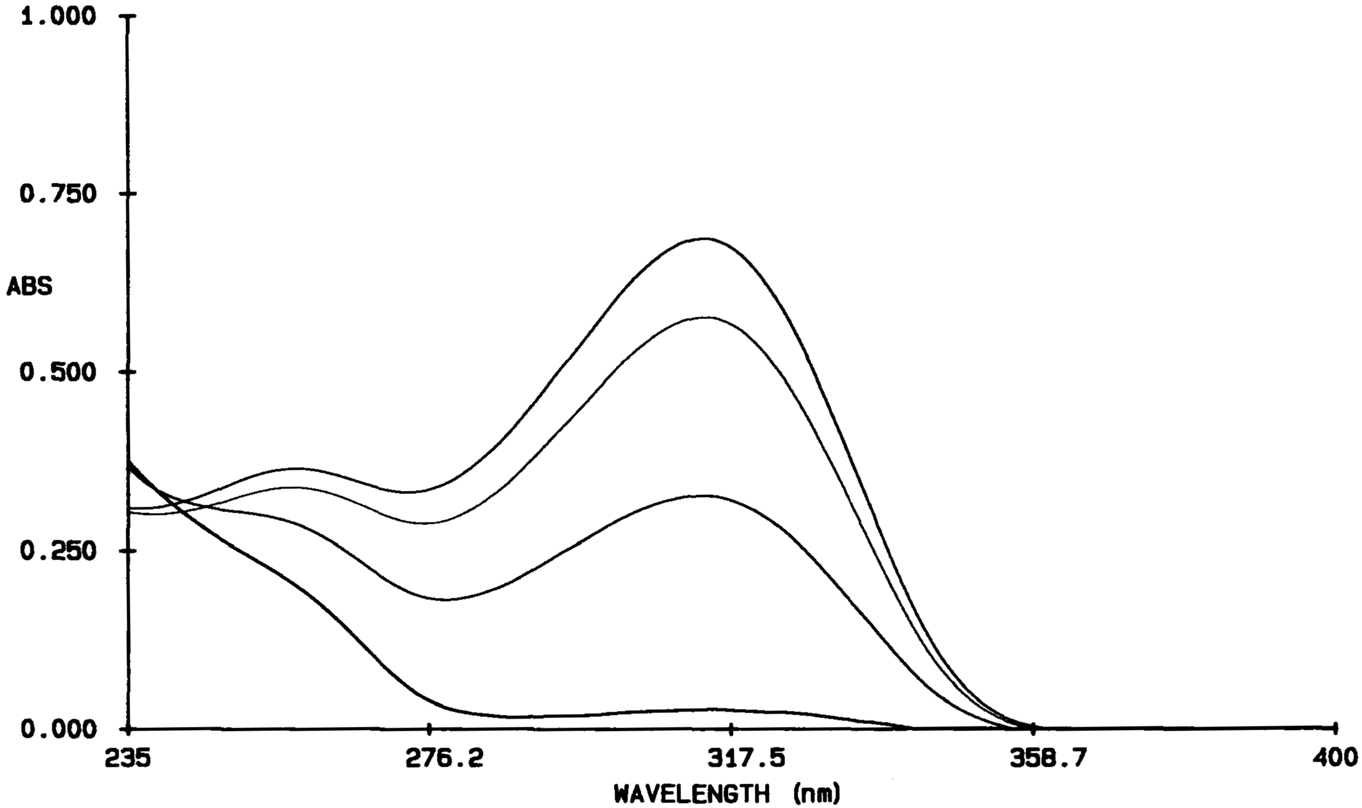


Figure 12. Ultraviolet absorption spectra of nizatidine, obtained at pH 1 (bottom), pH 2 (lower middle), pH 3 (upper middle) and pH 5 (top).

3.7 Thermogravimetric Analysis

The TGA thermogram for nizatidine, at a heating rate of 5°C per minute, shows no significant weight loss through 180°C. These data are consistent with nizatidine's properties as an anhydrous and non-hydroscopic material.

3.8 Crystal Properties, Polymorphism

The X-ray powder diffraction pattern of nizatidine is shown in Figure 13. The spectrum was obtained using a Nicolet powder diffractometer using copper Kα irradiation (1.5418 A) with a graphite monochrometer. A total of 21 peaks were detected at scattering angles between 5 and 35 degrees 2-theta. Table VI summarizes the data from X-ray powder diffraction where d is the interplanar spacing (A), and I/I_0 is the relative intensity of the X-ray line.

Nizatidine has been crystallized from solutions of ethyl acetate, butyl alcohol, methylene chloride and ethanol. X-ray diffraction studies indicate the presence of a single crystal form in all cases.

Table VI. Powder X-ray Diffraction Data for Nizatidine

D-Spacing (A)	Intensity (I/I_0)
12.59	0.39
6.36	0.08
6.11	1.00
5.98	.26
5.72	.11
5.46	0.20
5.25	0.17
4.43	0.15
4.38	0.04
4.18	0.48
3.98	0.30
3.85	0.43
3.71	0.35
3.63	0.15
3.42	0.28
3.36	0.13
3.33	0.43
3.22	0.28
3.07	0.15
2.99	0.15
2.88	0.13

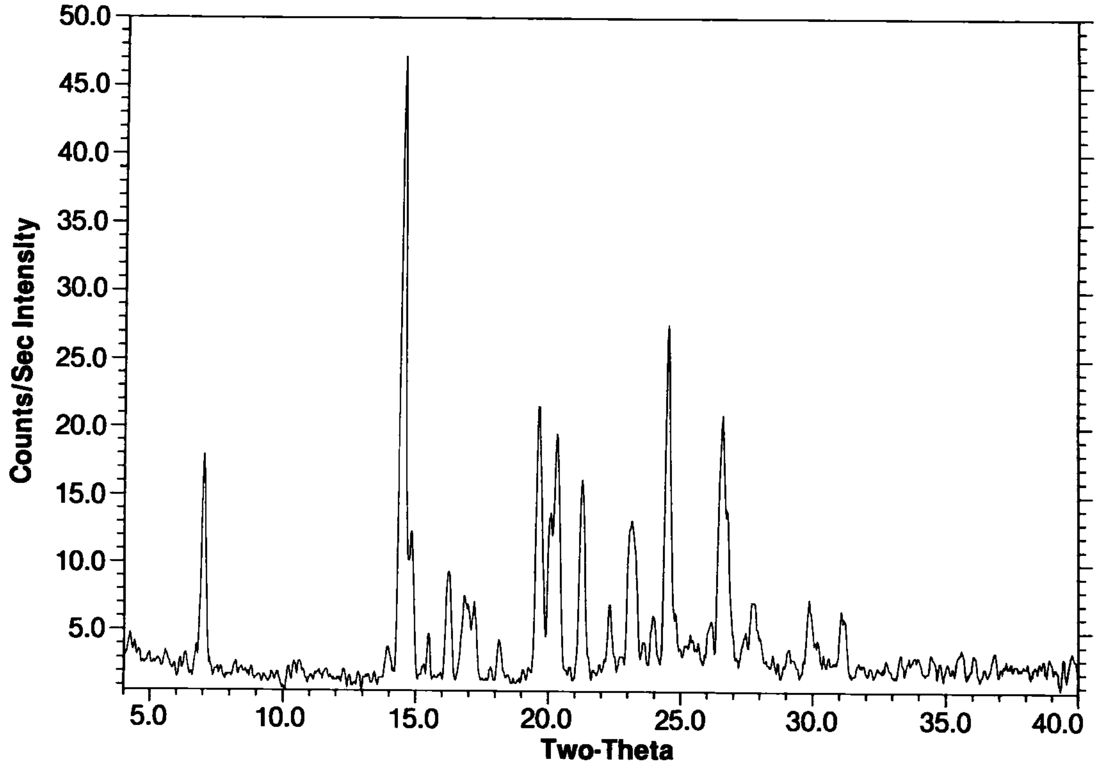


Figure 13. Powder x-ray diffraction pattern of nizatidine.

3.9 Solubility

Nizatidine is freely soluble in chloroform ; soluble in methanol; sparingly soluble in water and buffered solutions; slightly soluble in ethyl acetate and isopropanol. Nizatidine is essentially insoluble in benzene, diethyl ether and octanol. The solubility data for nizatidine are shown in Table VII.

Table VII. Solubility of Nizatidine

Solvent	Solubility (mg/ml)	USP Solubility
Water	≥10.0 - <33.3	Sparingly Soluble
Buffer - pH 7.0	≥10.0 - <33.3	Sparingly Soluble
Buffer - pH 4.5	≥10.0 - <33.3	Sparingly Soluble
Buffer - pH 1.2	≥10.0 - <33.3	Sparingly Soluble
Isopropanol	≥3.33 - <5.0	Slightly Soluble
Diethyl ether	<0.5	Very Slightly Soluble
Ethyl acetate	≥1.0 - <2.0	Slightly Soluble
Methanol	≥50.0 - <100.0	Soluble
Octanol	<0.5	Very Slightly Soluble
Benzene	<0.5	Very Slightly Soluble
Chloroform	≥100.0	Freely Soluble

3.10 Partition Coefficient

The octanol-water partition coefficient was determined to be 0.3 (octanol/buffer, pH 7.4), indicating that nizatidine exhibits little lipophilic character.

3.11 Ionization Constant, pK_a

The ionization constants in dimethylformamide were $pK_{a1} = 6.3$ and $pK_{a2} = 8.4$. The values for nizatidine in aqueous media were $pK_{a1} = 2.1$ and $pK_{a2} = 6.8$.

4. METHODS OF ANALYSIS

4.1 Identity

The identity of the nizatidine is determined using the specificity of infrared spectroscopy which differentiates it from any synthetic intermediates, process related substances or degradation products. Nizatidine is triturated with potassium bromide and pressed into a transparent pellet for spectroscopic analysis. The identity is confirmed by comparison to a reference standard spectrum obtained under similar conditions.

4.2 Elemental Analysis

The following elemental composition was obtained using a Perkin Elmer Model 2400 Analyzer.

Element	% Calculated	% Found
C	43.48	43.39
H	6.39	6.40
N	21.13	21.09
O	9.65	9.38
S	19.35	19.21

4.3 Ultraviolet

Nizatidine potency can be determined by ultraviolet spectroscopic assay in methanol at 325 nm, which avoids interference from known impurities and degradation products. The procedure is done at a concentration of 0.004 mg/mL in methanol using a 1 cm cell length.

4.4 Chromatography

4.41 Thin Layer

A TLC method can be used for the identity and purity of raw material for nizatidine. Precoated silica gel 60 F254 TLC plates are used as the stationary phase and a binary solvent system consisting of chloroform-methanol (98:2) is used as the developing solvent. Visualization is performed by exposing the plate to iodine vapors prior to viewing under short UV light (254 nm). This method will resolve known process related substances and degradation products from nizatidine.

4.42 High Performance Liquid

Conditions for quantifying nizatidine have been optimized using reversed-phase HPLC. The mobile phase consists of an ammonium acetate buffer (0.1M, pH 7.5 with 0.1% diethylamine) and methanol (76:24). A Jones Apex ODS column (150 x 4.6 mm; 5 micron particle size) is used in conjunction with a flow rate of 1 mL/min. Detection is obtained with an UV detector set at 254 nm. The method is stability-indicating as indicated by its ability to separate nizatidine and known degradation products. Figure 14 shows the separation of nizatidine and two known degradation products (nizatidine sulfoxide and nizatidine amide).

Gradient reversed-phase HPLC methodology is used to quantify nizatidine and its potential process related substances. A Supelco LC-18-DB column (250 x 4.6 mm; 5 micron particle size) is used in conjunction with a flow rate of 1 mL/min and detection at 254 nm. The mobile phase components are an ammonium acetate buffer (0.1M, pH 7.5 with 0.1% diethylamine) and methanol (76:24) (A), and HPLC-grade methanol (B). A linear gradient from 100% A to 66%A:34%B over 20 minutes is followed by a isocratic period of 25 minutes before recycle to 100% A. The sample is analyzed at a concentration of approximately 5 mg/mL.

The determination of nizatidine and its metabolites (des-methyl nizatidine and nizatidine sulfoxide) in plasma and urine is achieved by isocratic reversed-phase HPLC. The separation utilizes a Jones Apex ODS column (150 x 4.6 mm; 5 micron particle size) at a flow rate of 1.4 mL/min and detection at 313 nm. A Brownlee guard column (RP-8, 30 x 4.6 mm; 5 micron particle size) is used to minimize contamination of the analytical column. The mobile phase consists of an ammonium acetate aqueous portion (0.1M, pH 7.5 with 0.1% diethylamine) and methanol (76:24). The column temperature is maintained at 30°C by a thermostated column oven. Plasma and urine samples are pH adjusted with sodium bicarbonate prior to extraction with methylene chloride. Nizatidine and its metabolites are quantified versus a multi-point standard curve. Recoveries are corrected by use of N-[2-[[[-(dimethylaminomethyl]-4-thiazoyl]-methyl]thio]ethyl-N'-ethyl-2-nitro-1,1-ethylenediamine as an internal standard. Typical recoveries from both biological fluids are about 85 percent. Plasma samples were found to be stable for 3 months at 5°C and 6 months at -20°C. Urine samples were found to be stable for 2 weeks at 5°C and 1 month at -20°C.

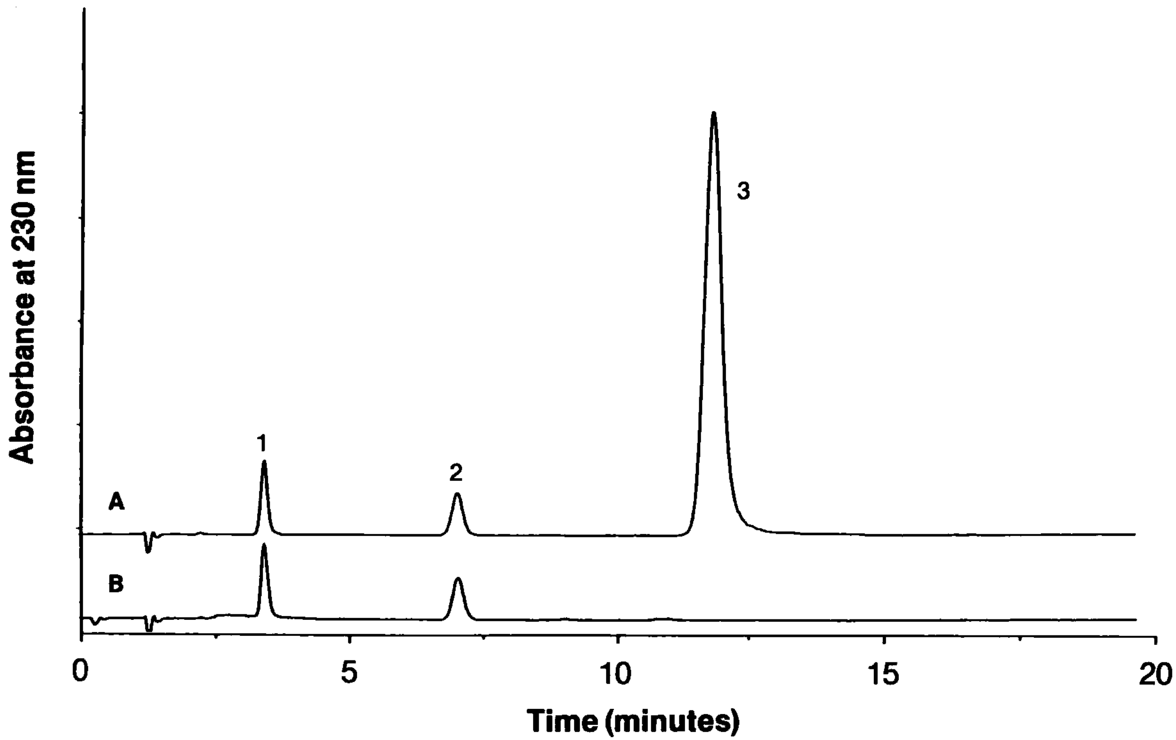


Figure 14. HPLC chromatogram of stability-indicating assay for nizatidine and degradation products (A), nizatidine degradation products only (B). Peak identification: (1) nizatidine sulfoxide, (2) nizatidine amide, (3) nizatidine.

5. STABILITY - DEGRADATION

5.1 Potential Routes of Degradation

Nizatidine bulk drug substance was found to be stable when exposed to thermal stress of 100°C for 7 days. The compound was also tested under harsh conditions of acid, base, peroxide and photodegradation. In all of these studies the compound was evaluated by specific HPLC and/or TLC procedures. The conclusions of these studies demonstrate that, while some decomposition was seen under extreme conditions, the nizatidine drug substance is very stable.

When exposed to the harsh conditions of heating under reflux for 24 hours in 1.0 N hydrochloric acid, nizatidine was found to be less stable. Three acid degradation products, 4-[[(2-aminoethyl)thio]methyl]-N,N-dimethyl-2-thiazolemethanamine (I), 2-[(dimethylamino)methyl]-4-thiazole-methanol (II), and 5,6-dihydro-3-(methylamino)-2H-1,4-thiazia-2-one oxime (III) were formed under these conditions (Figure 15).

Exposure to refluxing 0.1 N sodium hydroxide for 72 hours yielded three degradation products. These products, 4-[[(2-aminoethyl)thio]methyl]-N,N-dimethyl-2-thiazolemethanamine (I), N-methyl-2-nitroacetamide (IV) and N-(thiazoleamine)-2-nitroacetamide (V) are shown in Figure 15. One of the degradation products is common between both acid and base hydrolysis.

When exposed to a solution of 0.3% peroxide for 96 hours, nizatidine degraded to form nizatidine sulfoxide. The structure of N-[2-[[[2-[(dimethylamino)methyl]-4-thiazolyl]methyl]sulfinyl]ethyl]-N'-methyl-2-nitro-1,1-ethenediamine (VI) is shown in Figure 15.

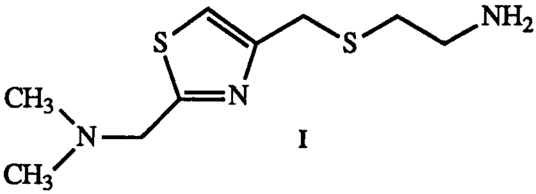
When irradiated with a mercury lamp for seven hours in a water solution, nizatidine degraded to N-[2-[[[2-[(dimethylamino)methyl]-4-thiazolyl]methyl] thio]ethyl]-N'-methyl urea (VII). This compound arises from the addition of water to the nitro-substituted double-bond followed by cleavage.

5.2 Solid-State Stability

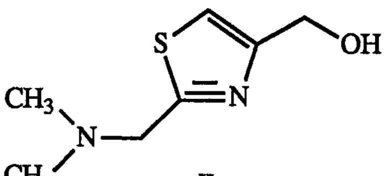
None of the degradation products produced through stress testing studies were found in lots of bulk nizatidine stored at room temperature for at least 48 months. Stability studies of the capsule formulation (150 mg and 300 mg dosage strength) were stable for at least 24 months at room temperature and 12 months at 40°C.

5.3 Solution Stability

Parenteral solutions of nizatidine have been shown to be stable for at least 24 months at room temperature. The primary degradation products in solution are nizatidine amide and nizatidine sulfoxide.



I



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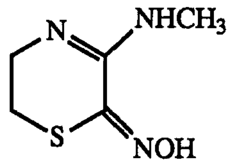
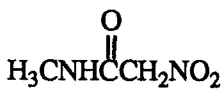
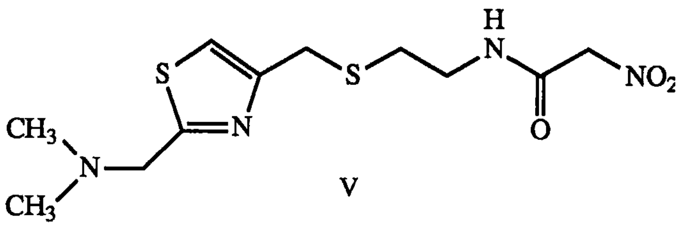


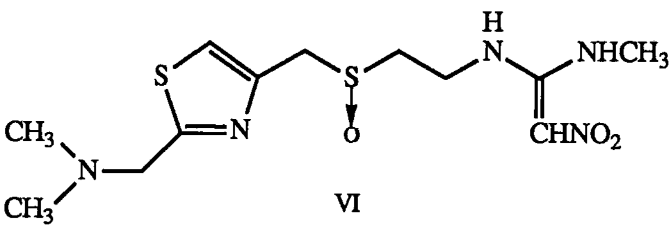
III



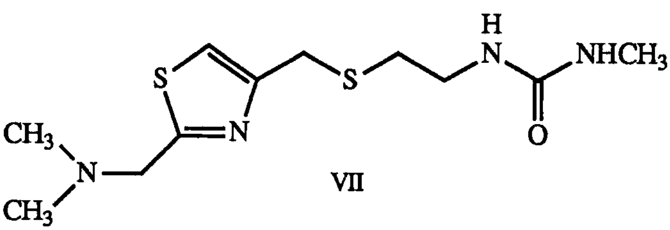
IV



V



VI



VII

Figure 15. Potential degradation products for nizatidine.

6. DRUG METABOLISM AND PHARMACOKINETICS

Metabolism and pharmacokinetics have been studied with ^{14}C-labelled nizatidine in the rat, dog, monkey and man (3, 10-18). In the rat and dog, nizatidine was excreted primarily in the urine as the parent drug. Nizatidine was metabolized to nizatidine N2-oxide in the dog, and to N2-monodesmethyl nizatidine in the rat (10). After oral administration in man, ^{14}C-labelled nizatidine was almost totally absorbed based upon the recovery in urine of over ninety percent of the radiolabel (11, 14, 18). Almost all of the radioactivity in urine is recovered within 16 hours. Unchanged nizatidine accounted for over sixty percent of the recovered dose. The three metabolites were identified as N2-monodesmethylnizatidine, nizatidine N2-oxide and nizatidine sulfoxide (11, 16-19).

Single doses of nizatidine 30, 100 and 300 mg inhibited gastric acid secretion by 57, 73 and 90 percent, respectively, for up to ten hours after ingestion (20). The mean decrease in acid secretion produced by these doses was significantly greater than that caused by placebo. In male hypersecretors of gastric acid, nizatidine inhibited gastric acid secretion for up to eight hours following intravenous infusion (21). In seriously ill patients at risk for stress gastritis, constant intravenous infusion was shown to be effective in maintaining gastric pH ≥ 4 (22-23).

The pharmacokinetic profile of oral and intravenous nizatidine were similar, with an elimination half-life of between 1.3 and 1.6 hours in healthy patients (10-11, 19). Increases in half-life were noted for patients with renal dysfunction (24-25). The similar pharmacokinetic profiles for both the oral and intravenous administration indicate that the disposition of nizatidine is largely independent of the route of administration. Peak plasma concentrations were achieved within one to three hours and increased linearly with dose. The absolute mean bioavailability of oral nizatidine has been determined with reference to the intravenous formulation and found to be in excess of 95 percent. The pharmacokinetics are unaffected by concurrent food ingestion; however the simultaneous administration of an antacid containing aluminum hydroxide gel and magnesium silicate decreased nizatidine absorption by ten percent (19). Unlike cimetidine and ranitidine, nizatidine does not inhibit the hepatic mixed function oxidase system in animals and man (3-6, 28-32).

The therapeutic efficacy of oral nizatidine has been investigated in patients with duodenal ulcer, benign gastric ulcer and gastro-oesophalgeal reflux disease (33-43). Nizatidine administered 150 mg twice daily or as a single bedtime dose of 150 or 300 mg was found to heal duodenal ulcers by the end of the clinical treatment period. The overall duodenal ulcer healing rates following eight weeks of treatment were similar to those of ranitidine (nizatidine, 91.8%; ranitidine, 92.5%) (42). In multicenter clinical trials for the treatment of benign gastric ulceration, nizatidine had an equivalent healing rate to that of ranitidine (nizatidine, 90%; ranitidine, 87%) (43). In a 12-week study of patients with gastro-oesophageal reflux disease a significant reduction in severity was noted for patients receiving nizatidine 150 mg twice daily (34-37).

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to the following people who have provided information for portions of this chapter: S.R. Maple and D.E. Dorman for the NMR spectra and interpretations; G.G. Cooke and J. Gilliam for the mass spectra and assignments; C.D. Underbrink and L.G. Tensmeyer for the infrared spectra and assignments; G.A. Stephenson for the x-ray diffraction spectra and assignments, D.R. Long for the thermal analysis data, and A.D. Kossoy for the pK/partition coefficient data.

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ANALYTICAL PROFILE OF RIBOFLAVIN

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Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

a) 3,10-Dihydro-7,8-dimethyl-10-[(2S, 3S, 4R)-2,3,4,5-tetrahydropentyl]benzopteridine-2,4-dione (1).

b) 7,8-Dimethyl-10-(1-D-ribityl)isoalloxazine (2,3).

c) 7,8-Dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl)Isoalloxazine (3).

d) 6,7-Dimethyl-9-(D,1′-ribityl)-isoalloxazine.

e) 3,10-Dihydro-7,8-dimethyl-10-(D-ribo-2,3,4,5-tetrahydroxypentyl)benzopteridine-2,4-dione (2).

1.1.2 Generic Names

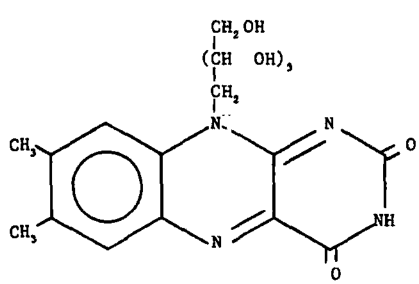
Vitamine G, Beflavine, Hepatoflavine, Lactoflavine, Lyochrome, Ovaflavine, Riboflavinum, Riboflav, Ribipea, Uroflavin, Vitamin B_2.

1.2 Formulae

1.2.1 Empirical

$C_{17}H_{20}N_4O_6$

1.2.2 Structural



Riboflavin

1.2.3 Cas Registry Number

83-88-5.

1.2.4 Wiswesser-Line Notation

T C666 BN DN
VMV 1NJ B1YQ
YQYA1Q LM

1.3 Molecular Weight

$C_{17}H_{20}N_4O_6$ = 376.4 (2)
376.36 (3)

1.4 Elemental Composition (3)

C 54.25%, H 5.36%, N 14.89%, O 25.51%.

1.5 Appearance, Color, Odor and Taste (2)

A yellow or orange yellow crystalline powder with a yeast like odor and a persistent bitter taste.

1.6 Optical Activity

The optical activity of riboflavin in neutral and acid solutions is exceedingly small. In an alkaline medium, the optical rotation is strongly dependent upon the concentration: $[\alpha]_D^{21} = -70°$ (c = 0.06%; 0.1 N NaOH); $[\alpha]_D^{21} = -117°$ (c = 0.5%; 0.1 N NaOH) (4). Borate-containing solutions are strongly dextrorotatory: $[\alpha]_D^{20} = +340°$ (pH 12); in this case the rotation depends only slightly upon the riboflavin concentration (5).

2. Physical Properties

2.1 Melting Range

About 280° with decomposition (2).

2.2 Solubility

Very slightly soluble in water (1 in 3000 to 1 in 20,000) of water (2). The variation in solubility being

due to the variation in the internal crystalline structure. The solubility of riboflavin in water only to an extent of 10 to 13 mg in 100 ml at 25° to 27.5°, 19 mg in 100 ml at 40°, and 230 mg in 100 ml at 100°. The vitamin (6) dissolves in ethanol to 4.5 mg% and is slightly soluble in amyl alcohol, cyclohexanol, benzyl alcohol, and phenol or amyl acetate. The impure material has a much higher solubility than the pure substances. Riboflavin is more soluble in physiological saline and in 10% urea solution. Practically insoluble in alcohol acetone, ether, chloroform or benzene. Formic acid dissolves more than 1% riboflavin.

For intravenous administration, sterile supersaturated solutions of riboflavin in normal saline could be employed (7). By heating to boiling and on exposure to sunlight, more than half of riboflavin is destroyed within 2 hours (8). The rate of destruction by light becomes higher with increasing temperature and pH. Alkali decomposes riboflavin rapidly.

2.3 Stability

Riboflavin is stable to acids, air and the common oxidising agents (except chromic acid, $KMnO_4$ and potassium persulphate), bromine and nitrous acid. The stability of riboflavin is used for the purification of the crude synthetic product; in acid solution impurities are oxidized at a temperature below 100° with use of Cl_2, H_2O_2, HNO_3 or $HClO_3$, but the vitamin is destroyed by hydrogen peroxide in the presence of ferrous ions (9).

2.4 pH

A saturated solution in water has a pH of 5.5-7.2 (1,2).

2.5 Occurrence (10)

Riboflavin is present in animal and plant cells but very few common food stuffs contain large amounts. Comparatively high concentrations occur in yeast cells and fermenting bacteria. Appreciable amounts are present in the liver (2-3 mgs/100 g), kidneys whole grain, dry beans, peas, nuts, milk, eggs and green

leafy vegetables. In tissues it occurs as FMN and FAD. Milk contains free riboflavin. Drying and cooking may increase the oxidisability of riboflavin.

Nutritional factor (3) found in milk, eggs, malted barley, liver, kidney, heart, leafy vegetables. Minute amounts present in all plant and animal cells.

2.6 X-Ray Powder Diffraction

The x-ray diffraction pattern of riboflavin was determined (Fig. 1) using Philips full automated x-ray diffraction spectrogoniometer equipped with PW 1730/10 generator. Radiation was provided by copper target (Cu anode 2000 W) high intensity X-ray tube operated at 40 KV and 35 MA. The monochromator was a curved single crystal one (PW 1752/00). Divergance slit and the receiving slit were 1 and 0.1° respectively. The scanning speed of the goniometer used was 0.02-2 θ per second. The instrument is combined with Philips PM 8210 printing recorder with both analogue recorder and digital printer. The goniometer was aligned using silicon sample before use. Values of 2 θ, interplaner distance dÅ and I/I_o X 100 are listed in Table (1).

2.7 Spectral Properties

2.7.1 Ultraviolet Spectrum

The ultraviolet spectrum of riboflavin (Figs. 2 & 2a) was scanned in 95% ethanol from 200 to 550 nm, using Pye Unicam PU 8800 spectrophotometer. The exhibited UV data is shown in Table 2.

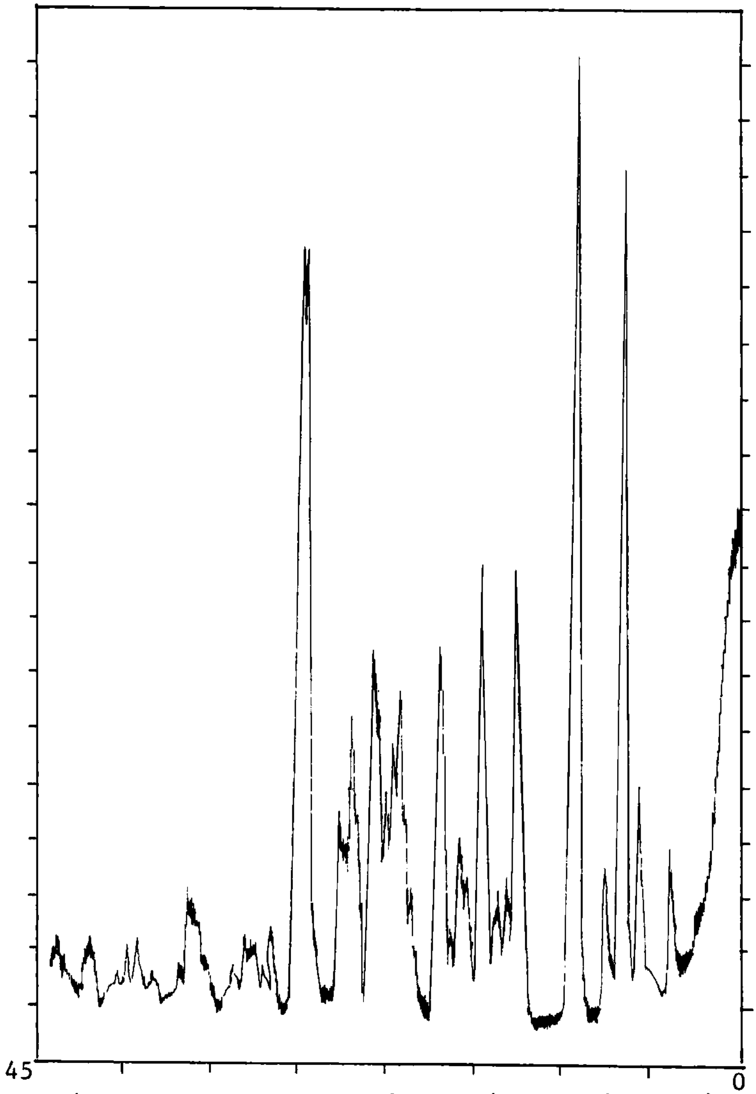


Fig. 1. X-Ray Powder diffraction of Riboflavin.

Table (1): X-ray diffraction pattern of riboflavin.

2 θ	dA	I/Io X 100	2θ	dA	I/Io X 100
5.269	16.7732	54.190	24.185	3.6799	36.823
5.518	16.0162	49.799	24.876	3.5792	26.886
7.209	12.2617	15.045	25.408	3.5055	33.898
8.677	10.1673	21.360	25.676	3.4694	40.004
10.454	8.4621	28.024	27.095	3.2908	23.914
11.572	7.6468	88.553	26.900	3.3143	33.991
12.369	7.1559	19.572	27.544	3.2383	24.494
14.328	6.1817	100.000	29.858	2.9923	51.056
17.526	5.0601	49.129	29.994	2.9791	82.029
18.006	4.9263	18.644	31.002	2.8845	12.096
16.491	4.7981	17.250	31.603	2.8310	12.955
19.392	4.5772	28.163	32.359	2.7665	12.421
19.550	4.5407	50.313	32.899	2.7224	12.630
20.281	4.3786	18.110	33.558	2.6704	10.033
20.660	4.2990	23.194	33.837	2.6490	8.381
21.135	4.2035	13.605	35.070	2.5587	10.030
21.847	4.0681	28.418	35.526	2.5269	14.488
21.924	4.0539	41.095	35.831	2.5060	16.043
23.420	3.7983	16.531	36.099	2.4881	16.624
23.871	3.7276	24.820	36.563	2.4575	10.076
24.185	3.6799	36.823	37.317	2.4096	8.637
24.514	3.6313	31.274	37.974	2.3694	9.217
24.876	3.5792	26.886	38.422	2.3428	9.705
25.408	3.5055	33.898	39.156	2.3006	13.396
25.676	3.4694	40.004	39.535	2.2794	11.214
27.095	3.2908	23.914	40.024	2.2526	9.333
26.900	3.3143	33.991	40.333	2.2361	9.031
27.544	3.2383	24.494	41.631	2.1693	10.448
29.858	2.9923	51.056	43.045	2.1013	10.378
29.994	2.9791	82.029	43.554	2.0779	12.026
31.002	2.8845	12.096			
31.603	2.8310	12.955			
20.281	4.3786	18.110			
20.660	4.2990	23.194			
21.135	4.2035	13.605			
21.847	4.0681	28.418			
21.924	4.0539	41.095			
23.420	3.7983	16.531			
23.871	3.7276	24.820			

dA = Interplanner distance.

I/Io = Relative intensity based on the highest as 100.

Table 2: UV characteristics of riboflavin.

λ_{max} nm	Abs	ϵ (Ethanol)
446	0.167	6279.2
358	0.129	4850.4
270	0.431	16205.6
222	0.393	14776.7

2.7.2 Infrared Spectrum

Infrared spectrum of riboflavin as KBr disc, was scanned on F.T.I.R., 1500 model Perkin Elmer Infrared spectrophotometer (Fig. 3). The structural assignments have been correlated with various frequencies in Table 3.

2.7.3 Nuclear Magnetic Resonance Spectra

2.7.3.1 PMR Spectra

The PMR spectra of ribolfavin in DMSO was recorded on a Varian T 60A, 60 MHz NMR spectrometer using TMS as internal reference. The spectra is shown in Fig. 4. The structural assignments have been reported in Table (4).

2.3.7.2 ^{13}C-NMR Spectra

The ^{13}C-NMR spectrum of riboflavin in DMSO-d_6 is obtained on a Joel 100 FT NMR spectrometer and is presented in Fig. (5).

2.7.4 Mass Spectrum

The mass spectrum of riboflavin is presented in the Fig. (6). The spectrum was obtained by electron impact ionization on a Varian MAT 1020 by direct inlet probe at 270°C. The electron energy was 70 eV. The spectrum was scanned from 100 to 400 a.m.a.

The spectrum Fig (6) shows a molecular ion peak M^+ at m/e 376 with a relative intensity of 10%. The base peak is 243 with a relative intensity 100%.

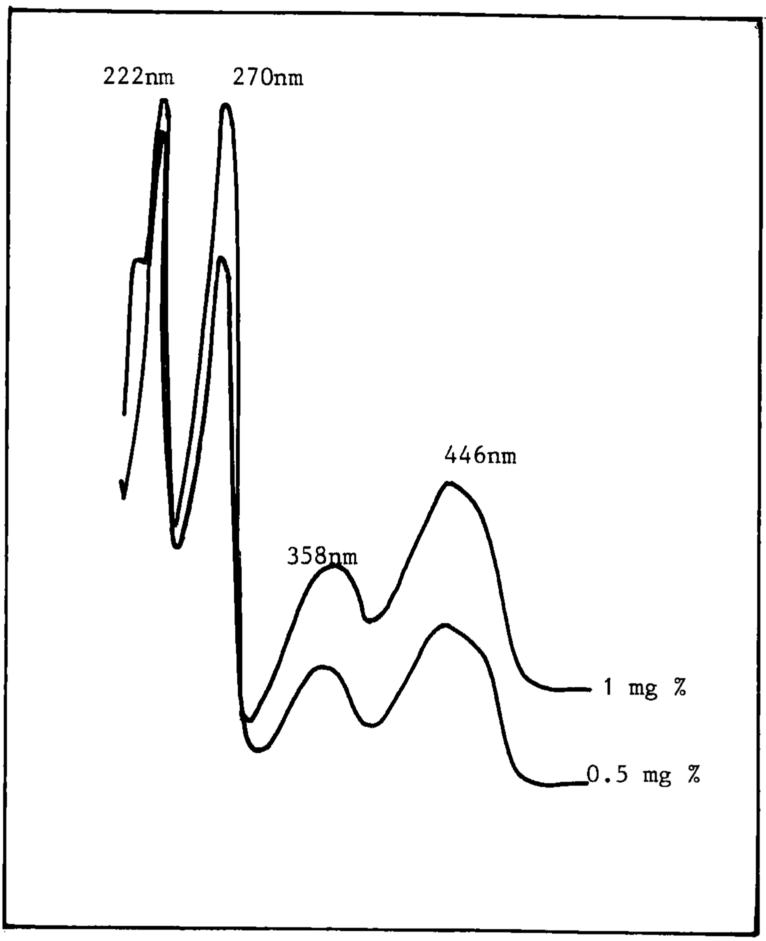


Fig. 2 UV Spectrum of Riboflavin in Ethanol.

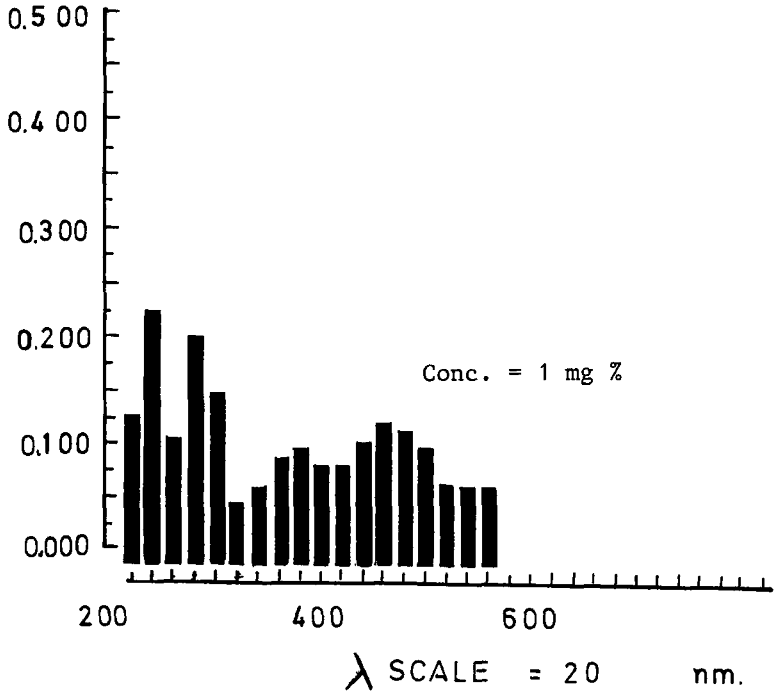


Fig. 2a. UV Spectrum of Riboflavin in Ethanol.

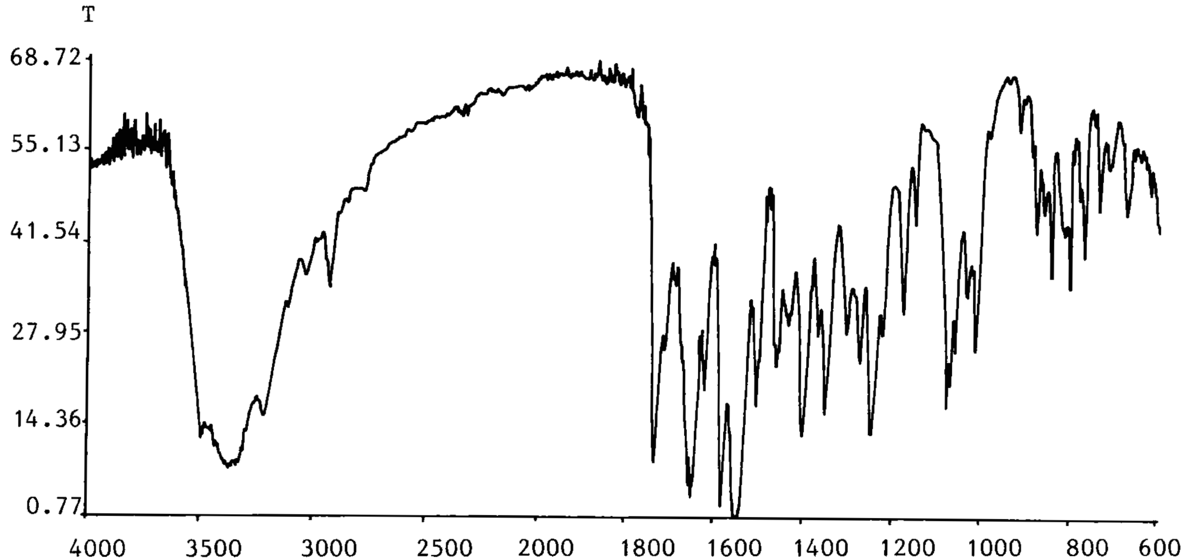


Fig. 3 I.R. Spectrum of Riboflavin (KBr disc.) Frequency cm^{-1}

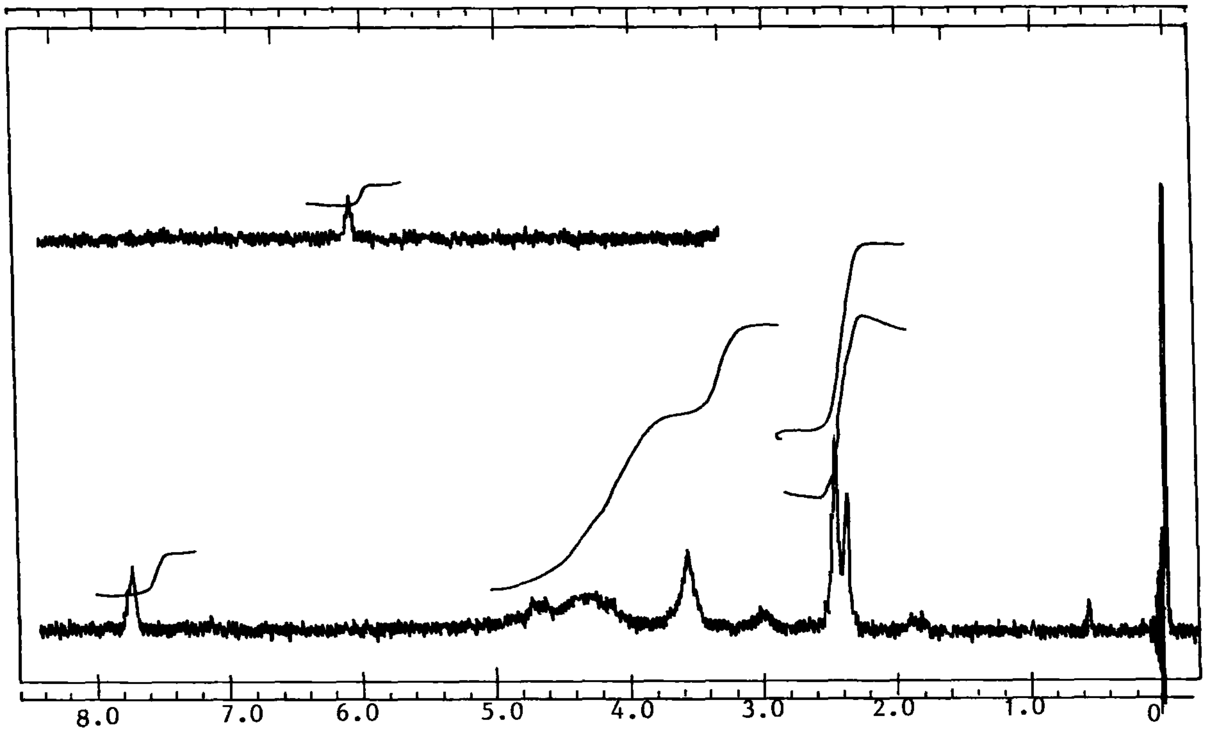
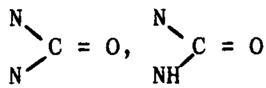


Fig. 4. PMR Spectrum of Riboflavin in DMSO-d_6.

Table (3): IR characteristics of riboflavin.

Frequency cm^{-1}	Assignment
3500, 3350 3210	N-H and O-H stretching, and possibly intramolecular hydrogen bonded -OH groups.
1730	
1650	C = O Diene, trienes; C = N -
1620	C = C ; - N - H
1580 1560	Aryl - H vibration frequencies.
1500 1450	- C - H deformations
1390	- CH_3 symmetrical deformation.
1340 1240	- O - H bending - O - H bending
1170 1070 1010	C - O stretching. C - O C - O
850 800 700	meta-disubstituted aromatic ring ortho-disubstituted aromatic ring due to -H, moving out of plane of the benzene ring.

The most prominant fragments, their relative intensities and some proposed ion fragments are given in the table (5).

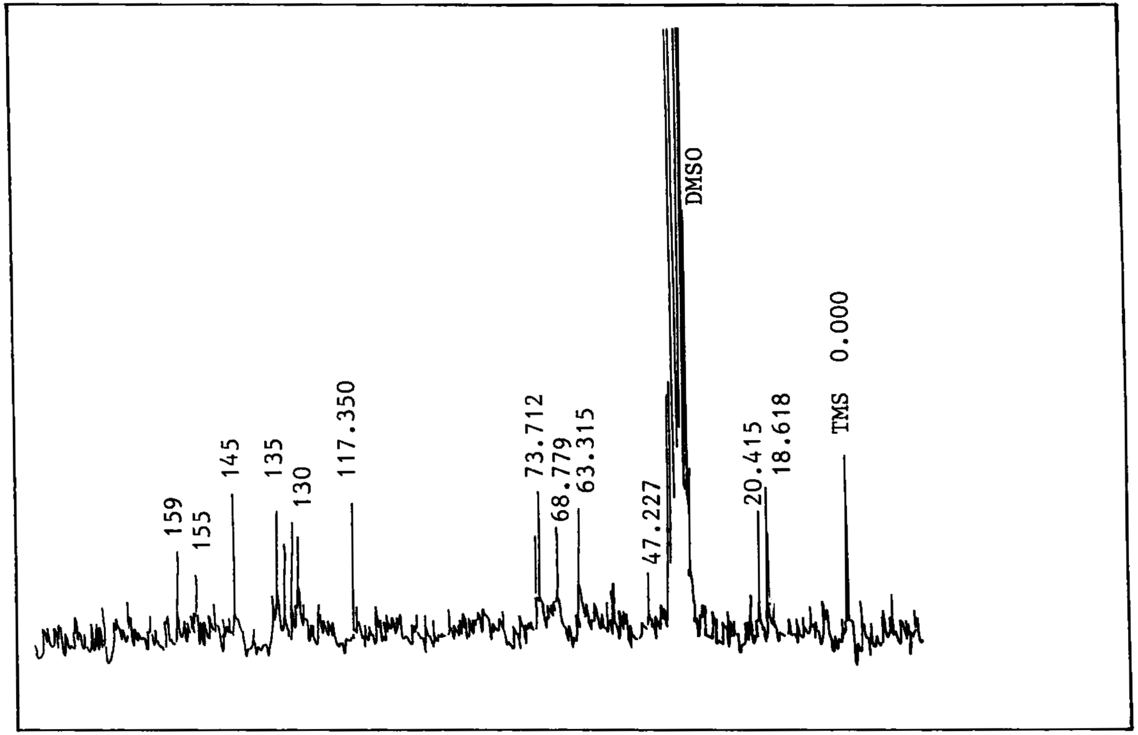


Fig. 5 ^{13}C-NMR Spectrum of Riboflavin in DMSO-d_6.

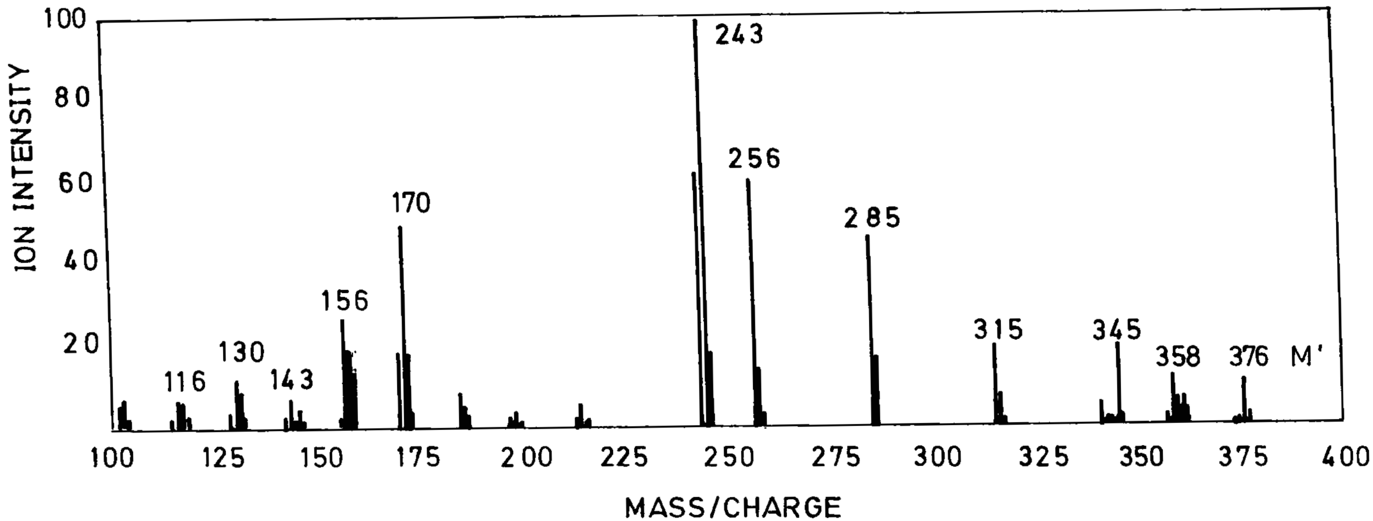


Fig. 6. Mass Spectrum of Riboflavin.

Table 4: PMR characteristics of riboflavin

Group	Chemical shift (δ) ppm
$2(CH_3)$	2.41 (m)
Aliphatic chain	2.8 - 5
Aliphatic-CH	5.03 (m)
Aromatic CH-CH	7.83 (s)
- NH	8.73 (s)

s = singlet, m = multiplet

3. Synthesis

Scheme No. I (11)

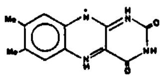
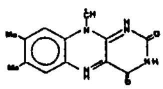
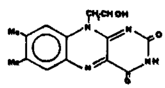
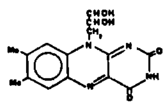
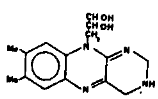
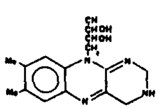
Some of the riboflavin is obtained from "distillery slope" formed in the alcohol determination of cereal grains. On commercial scale it is prepared synthetically. The basic material for the synthesis are 3,4-xylidine (4-amino-O-xylene), the sugar ribose, and barbituric acid or alloxan.

Xylidine is prepared by nitrating o-xylene and reducing the NO_2 in the resulting nitro compound to NH_2 by reduction.

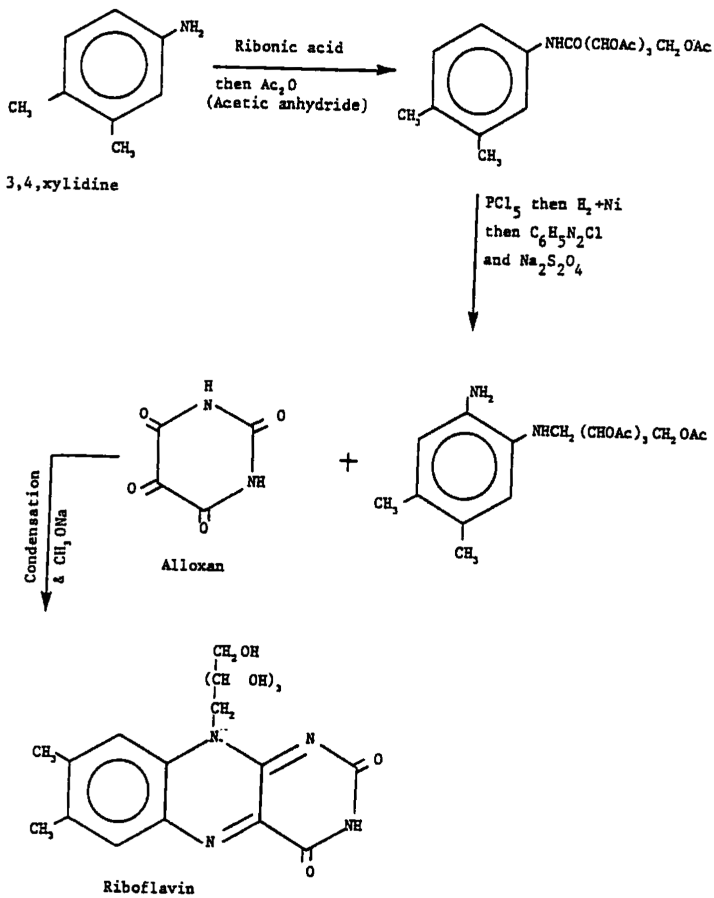
Ribose portion is introduced to the molecule by dissolving glucose in aq. KOH and is oxidized with oxygen to potassium salt of arabonic acid which is converted to calcium salt and treated with oxalic acid to get free ribonic acid.

Xylidine is heated with ribonic acid yeilding ribonoxylidine which is then acetylated by treating with acetic anhydride forming tetera-acetyl-ribonoxylidine then which is further treated with phosphorous pentachloride to get tetraacetyl-ribitylxylidine treating the teteraacetyl-

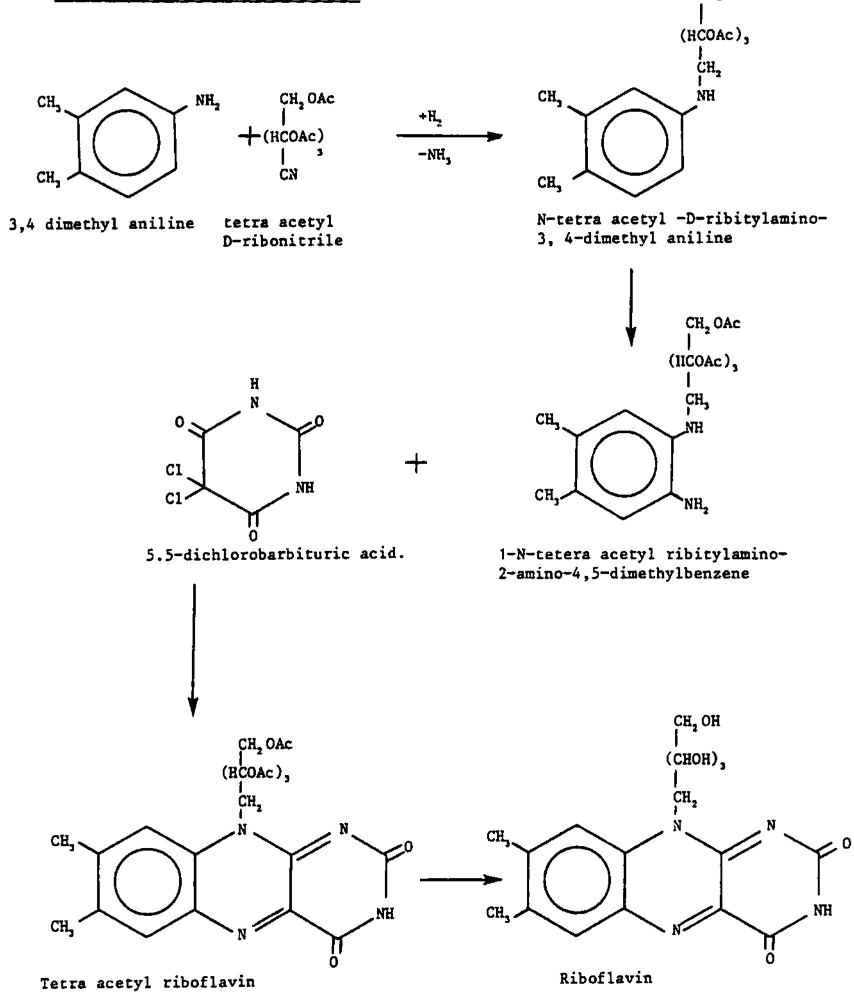
Table (5): The most prominent fragments of riboflavin.

m/e	Relative Intensity	Fragments
116	6%	OH OH OH CH-CH-CH-C-N^+
130	10%	O OH OH OH C-CH-CH-CH-C^+
243	100%	
256	60%	
285	44%	
315	18%	
345	18%	
358	12%	
376	10%	M^+ riboflavin.

Scheme I (Synthesis).



Scheme II (Synthesis).



ribitylxylidine with benzene diazonium chloride ($C_6H_5-N_2Cl$) or similar diazonium salt and then reducing with sodium hydrogen sulfite. When the resulting intermediate is reacted with alloxan or barbituric acid teteraacetyl riboflavin is formed. Acetyl group is removed by digesting with methanol containing a small amount of sodium methylate.

Alloxan can be prepared by oxidation of uric acid or barbituric acid with potassium permanganate or nitric acid.

<u>**Scheme II**</u> (12)

The method involves the reductive condensation of 3,4-dimethylaniline in the presence of palladium as a catalyst with tetraacetyl-D-ribonitrile with the loss of NH_3 gives N-tetraacetyl-D-ribitylamino-3,4-dimethylaniline. (Ribonitrile can be prepared from ribionic acid via the amide), the N-tetraacetyl-D-ribitylamino-3,4-dimethylaniline is coupled with p-nitrophenyldiazonium chloride, the product is reduced in the presence of platinum catalyst to 1-N-tetraacetyl-ribityl-amino-2-amino-4,5-dimethyl benzene. This compound is then condensed with 5,5-dichlorobarbituric acid to form tetraacetylriboflavin which is then hydrolysed to riboflavin.

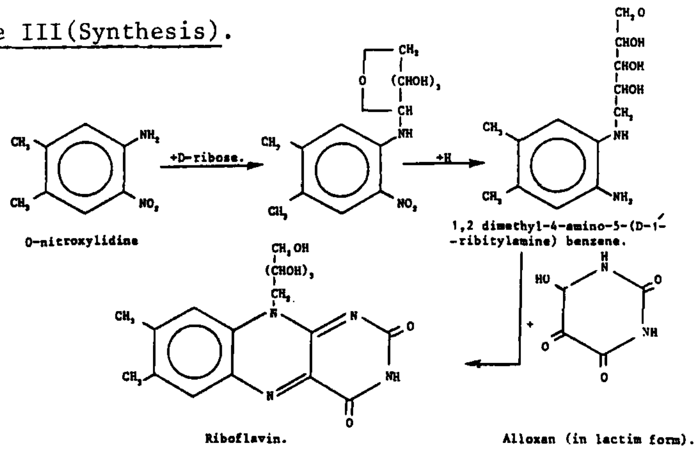
<u>**Scheme III**</u> (13)

In this method o-nitroxylidine is condensed with D-ribose to get 1,2-dimethyl-4-amino-(D-1′-ribitylamine) benzene and then it is condensed with alloxan which reacts in its lactim form.

The reaction takes place in acid solution by using the boric acid as a catalyst to increase the yield, other

catalyst which can be used are H_2S, $SnCl_2$ or alloxantin in the presence of 1 mol of HCl.

Scheme III(Synthesis).



4. Metabolism of Riboflavin

Riboflavin (2) is absorbed from the gastro-intestinal tract and in the circulation it is bound to plasma proteins. Although riboflavin is widely distributed, little is stored in the body, and amounts in excess of the body's requirements are excreated in the urine. Riboflavin in the faeces is probably the product of intestinal micro-organisms.

Riboflavin is converted in the body to flavine mononucleotide (FMN) and then to flavine adenine dinucleotide (FAD).

Patients with hepatitis and cirrhosis of the liver and those given probenecid had reduced absorption of riboflavin.

Renal clearance of riboflavine involved renal tubular secretion and exceeded endogenous creatinine clearance by up to 3 times. Clearance was reduced at low serum concentrations of riboflavine. Riboflavine was 60% bound to serum protein. Prior administration of probenecid decreased the renal clearance of riboflavine but did not effect its protein binding.

Riboflavin causes discoloration of the urine. A review chiefly from animal studies, of the effects hormones and drugs on riboflavine. Thyroid hormones, corticotrophin, and aldosterone, enhanced the formation of FMN while phenothiazines and possibly tricyclic antidepressants inhibited FAD formation. Boric acid increased the excreation of riboflavin.

In the single (14) whole lens of rat [^{14}C] riboflavin uptake increased in proportion to the substrate concentration in the inccubation medium, reaching a plateau at ≤ 60 min. At 4° the uptake was lowest, at 30°, 35° and 40° it was similar and higher than that at 4°. Neither uncouplers (2,4-DNP and monoiodoacetate) nor ovabain inhibited the uptake. In ≤ 15 min - 35% of transported [^{14}C] riboflavin was converted to ester forms of riboflavin, this ratio remained constant until 120 min incubation. Characteristically 2-3% of [^{14}C] riboflavin was bound to lens protein 2,4-DNP or monoiodoacetate inhibited the synthesis of ester forms of riboflavin but ovabain did not. No synthesis of ester forms of riboflavin, was observed in the lens capsule with epithelium. Apparently, 2 enzymes, flavokinase and FAD pyrophosphorylase, which convert riboflavin to FMN and FAD, respectively exist in the lens of the rat. These enzymes may participate in riboflavin uptake and riboflavin may pass through the lens capsule by simple diffusion without undergoing a conformational change. Riboflavin then may be metabolized to ester forms with subsequent partial binding to lens protein.

The effect of phenothiazine drugs and tricyclic antidepressants upon the conversion of riboflavin (15) into its active co-enzyme derivatives FAD. FAD was studied in rat tissues, chlorpromazine-HCl, phenothiazine derivative, imipramine-HCl and amitriptyline-HCl both tricyclic antidepressants, each inhibited the incorporation of [^{14}C] riboflavin into [^{14}C] FAD in liver cerebrum and heart. A variety of psychoactive drugs structurally unrelated to riboflavin were ineffective. Chlorpromazine, imipramine, and amitriptyline *in vitro* inhibited hepatic flavokinase, the first of two enzymes in the conversion of riboflavin to FAD.

5. Riboflavin Deficiency (Ariboflavinosis)

For the treatment of riboflavin deficiency (6) in adults, the usual oral dosage of riboflavin is 5-30 mg daily given in divided doses. For the treatment of riboflavin deficiency in children the usual oral dosage of riboflavin is 3-10 mg daily. The therapeutic response to the drug in riboflavin-deficient patients may not be dramatic. After several days, the occular and dermatologic manifestations of deficiency improve. In deficient patients with normocytic, normochronic anemia, an increase in reticulocyte count usually occurs within a few days following oral administration of 10 mg of riboflavin daily.

The vitamin deficiency (11) in animals is characterised by a reduced rate of growth, alopecia, dermatitis, cheilosis, cataract, bradycardia, and general collapse. Whereas deficiency in human the most frequently observed symptoms are cheilosis, a characteristic seborrhea, occular manifestations that include photophobia, conjuetivitis, and corneal vascularization.

The symptoms of human riboflavinosis include cheilosis (reddening of the lips and the appearance of tissues at the corners of the mouth, characteristic changes in the color of mucous membranes, inflammation of the tongue and denuding of the lips. Lessions of a seborrheic nature has also been observed in riboflavin deficiency. Occular manifestations that appear in man and animals are characterized chiefly by corneal vascularization, in which the cornea is invaded by small capillaries, which is usually accompanied by sensations of itching, burning and roughness of the eyelid, lacrimation, photophobia, and visual fatigue.

6. Human Requirements

The recommended daily dietary allowance (RAD) of riboflavin is 0.4-1.4 mg in children and 1.2-1.7 mg in adults (6). Requirements for riboflavin generally parallel the caloric intake or the metabolic body size. Oral riboflavin dosage of 1-4 mg daily are usually considered sufficient as a dietary supplement in patients with normal GI-absorption. During pregnancy

and lactation, riboflavin requirements are increased, however, adequate amounts of the vitamin are generally obtained from increased food intake in most of these women.

7. Uses

Riboflavin is used to prevent riboflavin deficiency and to treat ariboflavinosis. Whenever possible, poor dietary habits should be corrected, and many clinicians recommended administration of multivitamin preparations containing riboflavin in patients with vitamin deficiences since poor dietary habits often result in concurrent deficiences. (6)

Although an adequate amount of riboflavin is usually obtained from dietary sources, riboflavin deficiency may occur in patients with long standing infections liver disease, alcoholism malignancy and those taking probenecid. Increased riboflavin requirements may be associated with pregnancy and lactation or oral contraceptive use, however, riboflavin deficiency is rarely associated with these conditions. Although recommended daily dietary allowances (RDA) for riboflavin have been related to protein allowances, energy intake and metabolic body size, there is no evidence that riboflavin requirements are increased when energy utilization is increased.

Diagnosis of riboflavin deficiency (6) can be added by measuring erythrocyte or urinary riboflavin concentrations. Although these tests are not diagnostic, a urinary riboflavin conc. of less than 27-30 µg/g of creatinine, daily urinary excretion of less than 50 µg of riboflavin, or an erythrocyte riboflavin conc. of less than 10 µg/dL of erythrocytes is suggestive of deficiency. Corneal vascularization is another diagnostic sign. Occasionally, when a patients diagnosis is not clear, a trial of riboflavin may be used to diagnose riboflavin deficiency.

Riboflavin may be useful in treating mycrocytic-anemia that occurs in patients with a familiar metabolic disease associated with splenomegaly and glutathione reductase deficiency.

Because riboflavin is readily measured in urine, riboflavin (e.g. 2.5 mg) has also been mixed with various drugs as a marker to test for patient compliance with the therapeutic regimen of these drugs.

Although riboflavin has not been shown by well controlled trials to have any therapeutic valve, the drug has been used for the management of acne, migraine headache, congenital methemoglobinemia, muscle cramps, and burning teet syndrome.

8. Methods of Analysis

8.1 Elemental Analysis

The elemental analysis of riboflavin (3) as reported 376.36 is:

Element	% Theoretical
C	54.25%
H	5.36%
N	14.89%
O	25.51%

8.2 Identification Tests

a) Dissolve 1 mg in 100 ml of water (1). The solution has a pale greenish-yellow colour by transmitted light and by reflected light has an intense yellow-green fluorescence which disappears on the addition of mineral acids or alkalies.

b) Dissolve 50 (1) mg in 1.5 ml of carbonate free ethanolic potassium hydroxide solution and dilute with freshly boiled and cooled water to 10 ml. Specific optical rotation of the resulting solution is determined within thirty minutes of preparation - 115° to - 140°.

8.3 Titerimetric (16)

The formation of complexes of several vitamins with se\ n different picrates was studied, the riboflavin formed stable complex with Pb" or Zn picrates. The excess of picrate can be titerated with ethyleen tetraacetic acid solution and this fact has been used as the basis of a method for determining the composition of the complexes of Pb picrate with riboflavin.

8.4 Colourimetric

(i) To the 2 ml solution containing 1 ml to 15 µg of riboflavin add 5 ml methanol and 1 ml of 0.2 N NaOH and the mixture is diluted to 10 ml of the reagent solution. [Dissolve 0.2 gm of cupric chloride - triphenyl - phosphine complex in benzene (10 ml) and dilute the solution to 100 ml with methanol]. The extinction of the orange solution is measured at 460-465 nm. (17)

If the extraction of riboflavin is required, the reagent is prepared in butanol instead of methanol. In this case the 2 ml solution containing 10-70 µg of riboflavin is mixed with 1 ml of 0.2 N NaOH followed by 7 ml of the reagent solution.

After centrifugation 5 ml of organic extract is separated and diluted with 5 ml of methanol and its extraction is measured at 460-465 nm. (17)

(ii) The oxidation of riboflavin with periodate and formaldehyde produced is colourimetrically determined with chromotropic acid. To 0.2-0.8 ml solution containing 40-162 µg of riboflavin is diluted with 2 ml D. H_2O. 0.5 ml of 0.1 M sod. metaperiodate solution added and kept for 30 minutes in dark at room temperature. 0.5 ml of 5% sodium metabisulphite or sodium sulphite or sodium bisulphite is added to remove interferring iodate or periodate ions. To 1 ml of aliquot is mixed 5 ml of chromotropic acid, and heated at 100°C for 30 minutes. Coold and 0.5 ml of half saturated thiourea solution is added to remove the background colour. The contents are mixed and the

absorbance of resulting purple colour is measured at 570 mμ (18).

8.5 Spectrophotometric

1) Riboflavin is detected by (19) powdering the tablets and mixing with CH_3OH - CH_3ONa buffer at 90° for 10 minutes, filtering and then diluting two fold and measure the absorbance at 265.5 nm. Relative standard deviations were 50.8%.
2) To the injections (20) or tablets extract (1 ml ≡ 0.5 to 2.0 mg of riboflavin) add 20 ml of dioxane centrifuge for 10 minutes at 500 r.p.m. and decant the clear solvent layer. To the residue, add 0.2 ml of H_2O, 20 mg of nicotinamide and 10 ml of dioxane, centrifuge and repeat this operation until the extract contains no fluorescent colour. Treat the combine extracts with 5 ml of acetate buffer solution (prepared by adjusting a 10% solution of Na. acetate to pH 4 with anhydrous acetic acid) and dilute to give a final concentration of 1 mg of riboflavin per 100 ml. Measure the extinction at 444 nm against a reagent blank and calculate the amount of riboflavin in the sample assuming an E(1%, 1cm) valve of 323. Average recoveries were 95% with a coefficient of variation of 1.6%.

3) 5 Ml of sample (21) is shaken with about 12 mg of active carbon to adsorb the vitamin. The suspension is centrifuged and the solid is shaken with 5 ml of pyridine-acetic anhydride (4:1). The mixture is heated for 5 minutes in a water-bath at 60° to 70° then centrifuged and the fluorescence of riboflavin tetra-acetate in the supernatent solution is measured at 505 nm, with excitation at 448 nm. The final solution is stable at room temperature for about 24 hr and the calibration graph is rectiliner for up to about 25 $\mu g\ ml^{-1}$ of riboflavin.

4) 3 gm of yeast is extracted with hot H_2O (22) in an autoclave and the extract is deproteinised with sulphosalicylic acid, the ppt centrifuged off. The pH of supernatent liquid is adjusted to 7.0 with NaOH solution, the hexamine buffer solution of pH 7 is added and the mixture is transferred to a dark glass vessel. Then 0.2 M $AgNO_3$ is added. The solution is diluted to

known volume and after 10 min the absorbance is measured at 500 nm (4-cm cells) against H_2O. Beer's Law is obeyed for upto 5 µg ml^{-1} of riboflavin in the extract upto 30 µg ml^{-1} for pure solution.

5) To 75 mg of riboflavin add 150 ml of H_2O and 2 ml of glacial acetic acid and heat on a water bath with frequent shaking until dissolve (1). Cool and dilute to 1000 ml with H_2O. To 10 ml add 3.5 ml of 0.1 M sodium acetate and dilute to 50 ml with H_2O. Measure the absorbance of resulting solution at the maximum at about 444 nm. Calculate the contents of riboflavin taking 323 as the valve of A(1%, 1 cm) at the maximum at about 444 nm.

8.6 Polarographic Method

(1) Riboflavin can be determined (23) polarographically in multivitamins tablets, capsules and syrup. Dissolve the sample by boiling with 0.05 M KCl (100 ml) filter the suspension through paper, if necessary, add 0.2 M - KOH to bring the solution to pH 5.7-6.0 and dilute with 0.05 M - KCl to give a solution containing > 0.003% of riboflavin. Pass N_2 gas for 8 minutes and submit the solution to single-sweep cathode-ray polarography. Measure the peak height at about - 0.3 V for riboflavin.

(2) Another method (24) to determine the riboflavin by d.c. polarography from - 0.15 V vs the silver-AgCl reference electrode in 0.1 M phosphate buffer medium of pH 72; the optimum analytical concentration between 15-50 ppm. This method was directly applicable to two vitamin preparations but a third contained an interferring constituent (thought to be the coloring matter) this compound could be removed by ion-exchange or Dowex1-X8 resin (Cl^- form), with H_2O as eluent for the vitamin.

(3) In this method a solution of the tablets in alkaline Na citrate solution (pH 12) was analysed directly by polarography (25). Although insoluble additives interferred, their effect was constant for contents upto $\simeq$ 30%. The polarogram was recorded from - 100 to - 800 mV for riboflavin. The respective half-ware potentials (mV) was - 446. For 1-5 mg of

riboflavin in multivitamin tablets, the error was about ± 3%.

8.7 Fluorimetric

1) The method (26) involves the hydrolysis of the sample in trichloroacetic acid medium, separation of riboflavin from flavine mononucleotide on a column of florisil, with collidine buffer solution as eluent, and spectrofluorimetric determination of riboflavine in the eluate with use of the standard-addition method. The sensitivity is 0.01 µg ml^{-1} and 0.5 to 1.0 ml of sample is used. The normal level in 1-day old babies averaged 17.1 µg dl^{-1} and in women it was 14.2 µg dl^{-1} the contents in cord blood and maternal venous blood were also determined.

2) Samples of skimmed partially skimmed or homogenised milk and aq. ethanol extracts of non fat dried milk powder are deprotenised with acidified Pb acetate solution and riboflavin is determined directly in the clarified filtrates by use of a Turner fluorometer with a 110-812 primary filter (color specification 405) and 110-817 secondary filter (colour specification 8). There is a rectilinear relationship between fluorescence and riboflavin concentration upto 0.25 μg^{-1} or higher (r = 0.997). Recoveries of added riboflavin were 90 to 100 V with standard deviation of 1.71 to 3.16% (27).

3) The fluorimetric apoprotein titration of urinary riboflavin is done by the isolation and purification of the apoprotein (from chicken egg white) is as: the apoprotein solution in 0.05 - Tris buffer of pH 7.5 (1 mg ml^{-1}) was stored frozen in 2 ml aliquots. When required, each was thawed and diluted (1:3) with buffer solution. The diluted titrant was kept in an ice bath. The stock solution of pure riboflavin (18.9 mg in 500 ml of H_2O) containing 1 ml of conc. HCl) was diluted (1:199) with buffer solution to prepare a working standard. Portions of this standard were diluted to 10 ml (0.9 to 40 ng ml^{-1}) with buffer, 3 ml portions were placed in a fluorimetric cell, and the fluorescence at 520 nm was measured with excitation at 450 nm. Portions (10 µl) of titrant were added, and the fluorescence was measured after each addition, titrant

was added until three successive additions left the fluorescence unchanged (28).

Urine samples were diluted (1:9) with buffer solution and treated as for the standrds additional dilutions were made as necessary after the first addition (max. vol of titrant 150 µl). Recovery of added riboflavin averaged 98% the coefficient of variation ranged from 17% (for < 60 ng ml^{-1}) to $\simeq$ 2% (for > 200 ng ml^{-1}). Results for both standards and samples were generally higher than those by the assay based on lactobacillus casei.

4) By the use of physico-chemical method of analysis in quality control eye-drops. The method involves the measurement of the absorbance (blue filter) after diluting the sample with H_2O (29).

8.8 Solid-phase Enzyme-linked Assay

The assay of riboflavin is based on the competition between analyte vitamin molecules and a glucose-6-phosphate dehydrogenase-3-carboxymethyl riboflavin conjugate for a limited number of riboflavin-binding protein sites adsorbed on sepharose particles. Significant improvements in conjugate catalytic activity and thus detectability are possble by optimizing the reaction conditions used to covalently attach 3-carboxymethyl riboflavin to the enzyme. Optimization studies include, reaction, medium pH and organic solvent composition. Final assay detection limits and the sensitivity of the dose-response plots are also dependent on the ratio of conjugate to binding protein sites occupied in an equilibrium assay layout. Selectivity of the method correlates reasonably well with the predicted one, based on the known association constants of riboflavin-binding protein with flavin analogues. The assay gives reasonable detection limits for its determination in biological and drug samples (30).

8.9 Radioactive Isotopes

The method is competitive protein-binding assay for urinary riboflavin. It is suggested that the method may also be applicable to determine the vitamin in food

and pharmaceutical products. In this method 24-hr urine is collected in dark glass vessel containing 8 M-HCl and preferably referigerated; it should not be frozen, nor should it be stored for longer than one week. An aliquot of the urine is centrifuged (2000 r.p.m.; 5 min.) and the riboflavin is extracted into benzyl alcohol, with mechanical shaking for 10 seconds, the efficiency of extraction is about 83%. An aliquot of the extract is evaporated to dryness at 80° in a stream of N and an aq. solution of residue is allowed to equilibrate for one hour in a refrigerator with the protein solution (prepared from egg white) that has been saturated with ^{14}C-labelled riboflavin. Florsil is than added to adsorb free riboflavin and bound riboflavin is calculated by liquid scintillation counting of the supernatant solution. This method is specific and has a useful range of upto 500 µg of riboflavin in the aliquot analysed (31).

8.10 Competitive Binding Assays

A competitive binding procedure that can be used to determine either riboflavin or riboflavin-binding protein was developed. Riboflavin-binding protein from chicken egg white bind firmly to DEAE-cellulose while free riboflavin does not. Stock (2-14 C) riboflavin solutions, diluted with different amounts of a standard unlabeled riboflavin solution or an unknown sample, are mixed with aporiboflavin-binding protein and eluted through a small DEAE-cellulose column. The protein-bound riboflavin is batch eluted into scintillation vials, counted, and the unknown samples compared to a standard curve. This is a simple, rapid method for assaying riboflavin by isotope dilution. By a slight modification of the incubation conditions of this procedure, the degree of saturation and amount of riboflavin-binding protein can be determined. Data from both assays can be represented by linear plots in which slopes or intercepts correspond to unknown values (32).

8.11 Chromatographic Methods

8.11.1 Ion Exchange Paper Chromatography

The vitamin can be separated by using the ion-exchange paper 2. The strips (3 to 12 cm X 1 cm) of Amberlite papers SA-2 (H^+ form), WA-2 (NH_4^+ or Na^+ form) and SB-2 (OH^- form) are connected in series with adhesive cellulose tape, so that the solvent ascends from the top of the one strip to the bottom of the next. 0.2 to 5 mg of total vitamin are separated by using strips so connected, with H_2O or 0.2 M-Na acetate as solvent. The length of each strip and the order of connection vary with the composition of the test solution. Development takes 1 to 2 hrs with 0.5 ml of solvent (33).

8.11.2 Column Chromatography

(a) In this method 2 ml of an aq. solution containing 0.9 to 1.2 mg of riboflavin applied to a column (8 cm X 2 cm) of activated acidic alumina ($\simeq$ 22 g) suspended in $CHCl_3$-ethanol-acetic acid (500:500:3) and wash the column with the same solvent mixture until the yellow riboflavin band is near the base of the column. Elute the colour band with the same solvent mixture, collect the eluate, evaporate to 10 ml on a boiling water bath, cool and add about 20 ml of H_2O, and 5 ml of acetate buffer solution pH 4. Dilute the solution to 50 ml, with H_2O, mix, filter if necessary and measure the extinction at 444 nm. The recoveries ranged from 96.0 to 99.2% (34).

(b) Riboflavin was enriched by liquid solid extraction from large volumes of aqueous samples on a short pre-column. The enriched compound was transferred to an analytical reversed-phase column [25 X 0.41 cm packed with LiChrosorb Si 100. RPC18 (19% c wt./wt) with 0.005 M Na pentanesulfonate-0.005 M Na heptanesulfonate-MeOH (55:25:20) mix with 1 g H_3PO_4/L as eluant] and separated by ion-pair chromatography. The equipment used provides the possibility of automation for routine analysis (35).

8.11.3 Liquid Chromatography (LC)

A paired-ion liquid chromatographic technique coupled with flourometric detection has been used to assay riboflavin present in food stuff. Chromatograms of several such samples showed the presence of two distinct peaks of interest, due to the presence of riboflavin and flavin-mononucleotide (FMN). Relatively high levels of FMN were found in raw beef, corned beef, chicken liver and canned mushrooms. When riboflavin and FMN contents were summed up the liquid chromatograph values were comparable to those obtained by the AOAC standard procedures. However, the LC technique was simple, sensitive and rapid, yielding a mean standard deviation of 3.1% comparable to AOAC fluorometric method (3.0%) and better than the AOAC microbiological assay (9.6%). Mean spike recoveries were as follows (36):

LC	91.8%
AOAC (fluorometric method)	90.5%
AOAC (microbiological method)	89.6%

8.11.4 Thin-Layer Chromatography (TLC)

A summary of some of the TLC systems investigated for the analysis of riboflavin are given in the Table (6).

8.11.5 High Pressure Liquid Chromatography (HPLC)

High pressure liquid chromatography (HPLC) method has wide application for the estimation of riboflavin. A summary of variable parameters in a few cases is given in Table (7).

8.12 Extraction from Formulations

a) Extraction Procedure

The mixture of methyl alcohol, glacial acetic acid, pyridine and water in the ratio of (30:1:10:10) is used as extraction mixture. An accurately weighed amount of the sample is placed in a flask and the extraction mixture to the ten times of the dry weight of the sample is added. The flask is shaken well and refluxed for one hour and then cooled. If lumping is observed,

Table (6): Summary of conditions used for the TLC of riboflavin

Support	Solvent system	Detection	Extd. solvent	Ref.
0.2 mm silica gel	Pyridine: acetic aicd : H_2O (19:2:79)	Fluorescence	-	37
Fertigfolien	D. H_2O	Expose dried chromatogram to Cl then spray o-tolidine K_1 reagent. U.V. 254.		38
0.4 mm layer cellulose-silica gel GF_{254} (2:1)	$CHCl_3$: ethylether: ethanol: acetone: H_2O (4:12:10:6:3)	U.V.	0.1 N HCl (4 ml)	39
Silica gel	Toluene: methanol: acetic acid (10:9:1)	U.V.	-	40

Table (7): Summary of HPLC conditions for the determination of riboflavin

Column	Mobile phase	Flow rate ml/min	Detection	Sample	Ref.
Radial Pak A cartridge (C18)	Phosphate buffer solution methanol (7:3)	2 ml/min	Fluorimetric 450 nm	Urine	41
Column (10 cm X 18 mm) ID, radial Pak C8 (10 µm) and guard column of Bondapak C18/Porasil.	3.7% methanol, 0.1 M phosphate buffer (pH 7.0)	1.5 or 3 ml/min	Flourimetric 530 nm	Food	42
Nucleosil C18 (5 µm)	0.01 M phosphate buffer pH 5.0, methanol (13:7).	-	Flourimetric	Blood	43
µ Bondapak C18	3 to 8 mM Na hexane sulphonate in aq. 25% methanol containing 1% of acetic acid.	1 ml/min	254 nm	Powdered tablets or injections	44
Lichrosorb NH2	Methanol: Na acetate buffer pH 4.5 (1:1).	-	Spectro-fluorimetric 525 nm	Food	45

Continued /...

Continued Table (7)...

(30 cm X 3.9 mm) of µ Bondapak phenyl (10 µm).	0-80% of methanol in H_2O	2 ml/min	U.V. 280 nm	Multivitamin tablets	46
(30 cm X 4 mm) of µ Bondapak C18	0.2 M acetate buffer with 5 mM heptane sulphonic acid.	1 ml/min	U.V. 250 nm	Food	47
Stainless steel column (50 cm X 2.1 mm) packed with Spherisorb silica (20 µm).	$CHCl_3$: methanol (9:1)	1.0 ml/min or 0.8 ml/min.	Flourimetry 270 nm	Meat	48
Stainless steel column (30 cm X 4 mm) packed with µ Bondapak C18 (10 µm).	Methanol-aq. 5 mM hexane-sulphonate containing 1% of acetic acid (1:3)	0.5 ml/min	U.V. 270 nm	Multivitamin	49
Two columns (50 cm X 2.1 mm) connected in series and packed HS Pellionex Scx.	0.025 M HNO_3	10-20 ml/hr.	U.V. 254 nm or 280 nm	Multivitamin	50

Continued /...

Continued Table (7)...

(250 X 4 mm) Nucleosil 100-C18 with (7.5 X 4.5 mm) C18 guard column.	65:35 Isocratic mix of 0.1% NaH2PO4, pH 3, containing 0.005 M heptanesulphonic acid + MeOH containing 0.005 M heptanesulphonic acid.	-	Flourometrically (375/525 nm)	Milk	51
Zorbax-NH2	MeOH - 0.2 M phosphate buffer.	-	Fluorometric	Serum	52
(4 ft X 0.12 in) with Lichrosorb S160	CHCl3: methanol: acetate buffer solution (120:56:9)	0.95 ml/min	U.V. 254 nm	Multivitamin	53
Aminex A-5 resin	0.03 M - KCl in 0.1 M K phosphate buffer pH (8.0).	-	-	-	54
(7 cm X 2.1 mm) of Co: Pell ODS, and (30 cm X 4 mm) µ Bondapak C18 (10 µm).	0.01 M KH2PO4 (pH 5): methanol (13:7).	2 ml/min	Spectroflourimetric	Urine	55

Continued /...

Continued Table (7) ...

(25 cm X 4 mm) with 1EX-540	0-0.05 M KH_2PO_4	-	Fluorimetry	Tissues	56
Guard column (4 cm X 4 mm) Spherisorb ODS + (15 cm X 4 m) Magnusil C22.	0.1 M trisodium citrate: 0.1 M citric acid: H_2O: methanol (191:59:50:200).	-	Fluorimetry 520 nm	Food	57
(30 cm X 3.9 mm) of µ Bondapak C18 + guard column of Bondapak C18/Corasil.	Aq. 30% methanol.	1 ml/min	Fluorimetry 530 nm.	Urine	58
Precolumn (11.5 cm X 1.5 mm) with Corasil C18 (37-50 µm) + (25 cm X 4 mm) Zorbax PB-TMS (7 µm).	Aq. acetate buffer (pH 4.5) + acetate buffer: methanol (13:7).	-	Flourimetry > 460 nm	Haemodaly-sate	59
(25 cm X 4 mm) 1EX-540.	Licl in aq. 30% acetonit-rile	-	Flourimetry	Serum or blood	60

Continued /...

Continued Table (7) ...

(30 cm X 3.9 mm) μ Bondapak C18 + pre-column (2 cm X 4 mm) of Bondapak-Corasil (37-50 μm).	10 mM-diammonium hydrogen: phosphate (pH 5.5 and H3PO4): acetonitrile (25:3).	2 ml/min	254 nm	Plasma	61
Bondapak C18	Water: methanol: acetic acid (68:21:0.1 vol/vol).	1.0 ml/min	U.V. detect	-	62

the mixture is agitated. It is diluted to a known volume with the extracting mixture, keeping in mind that the concentration of ribolfavin in the assay solution should not fell below the sensitivity of the method to be used for analysis. For fluorometric method, the concentration of ribolfavin should be 0.1 µg/ml. The solution is filtered. The procedure has been used for the extraction of riboflavin in multivitamin tablets, capsules, liquids and mineral dietary supplements (63).

b) **Adsorption on Purified Talc**

A column 12 X 200 mm, with a stop clock connected to a suction pump is employed and a slurry of talc having a particle size 20-50 µ in water, sufficient to form about an 80 mm bed is poured into the column. A flow rate of about 4-6 ml/minute is maintained by adjusting the suction. The diluted sample is allowed to pass through the talc column followed by a sufficient volume of 0.01 N HCl and 10% Dioxan in order to elute other constituents adsorbed on the talc. The yellow-coloured band of riboflavin adsorbed on the talc bed from the assay sample is then eluted with 10 ml of 20% dioxan. The determination of the amount of riboflavin thus eluted by a spectrophotometric method by the diluting the above solution to a volume containing 1 mg/100 ml and then measuring the absorbance of the yellow colour at 267, 374 and 445 mµ (64).

8.13 Thermal Analysis (DSC)

A DSC (differential scanning calorimetry curve) of riboflavin was obtained Fig. (7) on a Perkin Elmer DSC-2C differential calorimeter. The analysis was conducted under N_2 atmosphere, at a scan rate of 25°C/min. The DSC curve revealed an endothermic melting peak max. 309.61°C.

9. Acknowledgements

The authors are highly thankful to Mr. Tanvir Ahmad Butt, Pharmaceutical Chemistry Department, College of Pharmacy and Mr. Babkir Awad Mustafa, College of Applied Medical Sciences, King Saud University, Riyadh

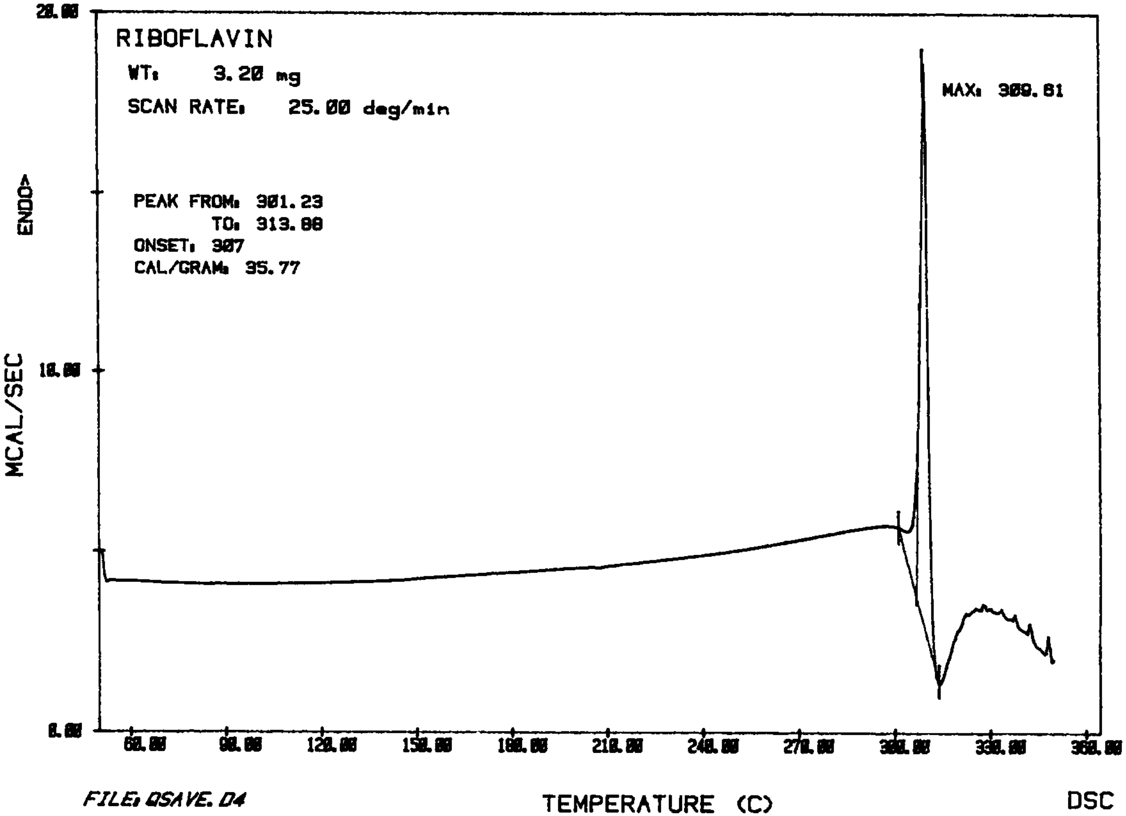


Fig. 7. Thermal curve of Riboflavin.

for their sincere help in the preparation of this manuscript.

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ANALYTICAL PROFILE OF SCOPOLAMINE HYDROBROMIDE

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SCOPOLAMINE HYDROBROMIDE

Contents

1. Description

1.1 Nomenclature

1.1.1 Chemical Names

a) α-(Hydroxymethyl) benzeneacetic acid 9-methyl-3-oxa-9-azatricyclo $[3.3.1.0^{2,4}]$ non-7-yl ester.
b) 6β, 7β-epoxy-1αH, 5αH-tropan-3α-ol (-)-tropate.
c) 6β, 7β-epoxy-3α-tropanyl S(-)-tropate.
d) 6,7-epoxytropine tropate.
e) Benzeneacetic acid, α-(hydroxymethyl)-, 9-methyl-3-oxa-9-azatricyclo $[3.3.1.0^{2,4}]$ non-7-yl ester, [7(S)-(1α,2β,4β,5α,7β)].
f) (-)-(1S,3s,5R,6R,7S)-6,7-Epoxytropan-3-yl(s)-tropate.

Example for the hydrobromide salt:
Benzeneacetic acid, α-(hydroxymethyl)-,9-methyl-3-oxa-9-azatricyclo $[3.3.1.0^{2,4}]$ non-7-yl ester hydrobromide, [7(S)-(1α,2β,4β,5α,7β)].

1.1.2 Generic Names

Scopolamine hydrobromide; Hyoscine hydrobromide; Scopolammonium bromide; Scopolamine bromohydrate; Escopine tropate hydrobromide; Tropic acid ester with scopine as the hydrobromide.

1.1.3 Trade Names

Transderm Scop (forl-scopolamine) Joy-rides; Quick K wells; Scopos; Sereen (for scopolamine hydrobromide).

1.2 Formulae

1.2.1 Empirical

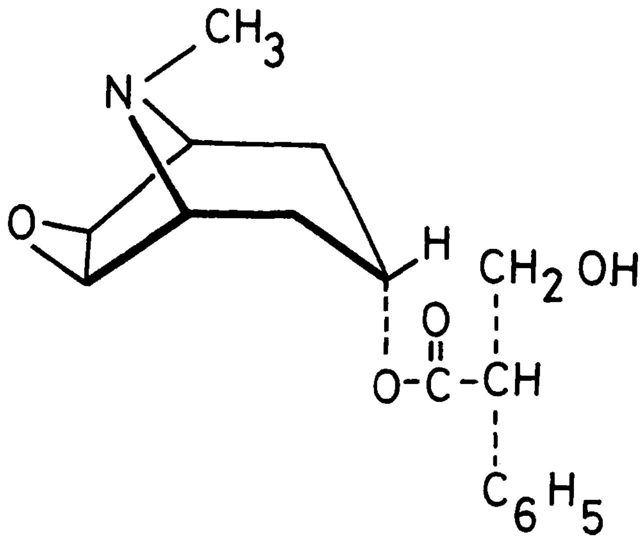
$C_{17}H_{21}NO_4$ (scopolamine)

$C_{17}H_{22}Br\ NO_4$ (scopolamine hydrobromide)

1.2.2 Structural

The structure of scopolamine has been confirmed by total synthesis, which was achieved

by several authors (1-4).



1.2.3 CAS Registry Number

[51-34-3] Scopolamine

[114-49-8] Scopolamine hydrobromide

1.2.4 Wiswesser Line Notation (5)

TC 356 A AND OTJ A HOVYR & 1Q
*DXLV (Scopolamine)
TC 356 A AN D OTJ A HOVYR & 1Q
& EH *DXLV (Scoplamine hydrobromide dl).

1.2.5 Stereochemistry

The stereochemistry of scopolamine has been determined by chemical means as well as the X-ray crystalographic study of the hydrobromide salt (6). It is established that natural (-)-scopolamine has the (S)-configuration *(by relationship with (-)-tropic acid which has been shown to possess the (S)-configuration)* (7).
The structure of (-)-(S)-scopolamine hydrobromide revealed the conformation of the tropine residue to be as expected, with the

six-membered ring in the chair conformation. The epoxide oxygen atom on the methylene bridge is in the boat configuration with respect to the nitrogen atom, and the methyl group C(1) attached to the nitrogen atom is in the *axial* position with respect to the six-membered ring *(unlike the equatorial configuration of this methyl group as found in cocaine hydrochloride (* 8 *), tropine (* 9 *) and pseudotropine (*10).
The ester group attached to C(13) is in the axial (α) position of the six-membered ring. *The structure of (-)-(S)-scopolamine hydrobromide is shown in Fig. 1 (* 6 *).*

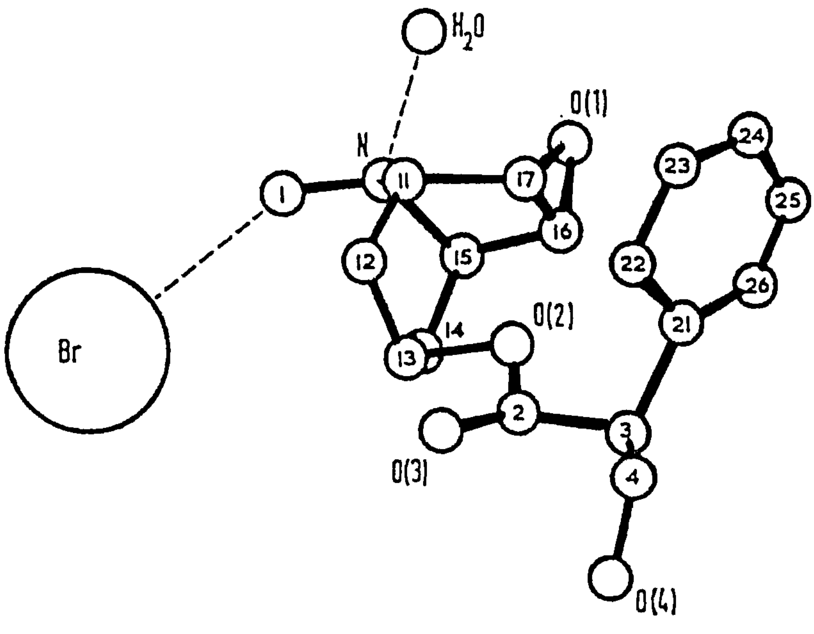


Fig. 1 : The structure of (-)-(S) scopolamine hydrobromide.

1.3 Molecular Weight

303.35 (Scopolamine)
384.30 (Scopolamine hydrobromide)

1.4 Elemental Composition

C, 67.31%; H, 6.98%; N, 4.62%; O, 21.10% (Scopolamine).
C, 53.13%; H, 5.77%; Br, 20.80%; N, 3.65%; O, 16.65% (Scopolamine hydrobromide).

1.5 Appearance, Color and Odor

- Scopolamine occurs as a viscous liquid, colorless, odorless. It forms a crystalline monohydrate (11).
- Scopolamine hydrobromide occurs a rhmobic crystals or as a white crystalline powder, slightly efforescent in dry air (12 ,13), odorless and has a very bitter taste (13).

2. Physical Properties

2.1 Melting Range

59° scopolamine monohydrate (11).
The following melting range has been reported for scopolamine hydrobromide:

Melting Range	Reference
197-200° (after drying at 105°)	(13)
Between 195-199° (after drying at 105°)	(14)
At about 197° (with decomposition)	(12)
195°	(11)
193-194°	(15)

2.2 Eutectic Temperature

The eutectic temperature of scopolamine hydrobromide is recorded as follows (13):

	Microscope Hot Stage	Hot Bar
Sal.	165°	168°
Dic.	126°	130°

Sal. = Acetaminosalol; Dic. = Dicyandiamide

2.3 Solubility Data

- Soluble in 9.5 parts of water at 15°, freely soluble in hot water, in alcohol, ether, chloroform, acetone. Sparingly soluble in benzene and petroleum ether (scopolamine) (11).

- One gram dissolves in 1.5 water, 20 ml alcohol. Slightly soluble in chloroform and almost insoluble in ether (scopolamine hydrobromide) (11).
  The B.P. (12) reported the following solubility data for scopolamine hydrobromide: soluble in 3.5 parts of water and in 30 parts of ethanol (96%); practically insoluble in chloroform and in ether.

2.4 pH Range

Between 4.0 and 5.5 in a solution (1 in 20) (12,13).

2.5 Specific Optical Rotation

Scopolamine $[\alpha]D^{20}$ - 28° (C=2.7) (11)

$[\alpha]D$ - 18° (EtOH) (15)

Scopolamine hydrobromide:

$[\alpha]D^{25}$ - 24° to - 26° (C=5) (11,14)

$[\alpha]D$ - 24° to - 27° (12)

$[\alpha]D$ - 25.9° (H_2O); $[\alpha]D$ - 15.72° (EtOH) (15)

2.6 Dissociation Constant

pKa = 7.6 (23°) scopolamine hydrobromide (16).

2.7 Crystal Structure

The crystal structure of (-)-(S)-scopolamine hydrobromide as $C_{17}H_{21}NO_4$, HBr is reported (6). The crystals are colorless needles, elongated along C, Lane group P4/mmm, space group $D_4^6 — P4_22_12_12$, a= 1196.5±0.7, c=2652±2 pm, Z=8. One half molecule of water per molecule of scopolamine has been found crystallographically (6).
The distances of the nitrogen atom from the various atoms of the molecule are: N-O(1) 247; N-O(2) 388, N-O(3) 541 and N-O(4) 804 pm.
The molecule of (-)-(S)-scopolamine hydrobromide projected down is shown in Fig. 1.

2.8 X-Ray Powder Diffraction

The X-ray diffraction pattern of scopolamine hydrobromide was determined with a Philips X-ray diffraction spectrogoniometer equipped with PW 1730 generator.

Radiation was provided by a copper target (Cu anode W, γ= 1.5480 A°). High intensity X-ray tube operated at 40 KV and 35 MV was used. The monochromator was a curved single crystal (PW 1752). Divergance slit and the receiving slit were 0 and 0.1° respectively. The scanning speed of the goniometer used was 0.02 2θ per second.
The X-ray pattern of scopolamine HBr is presented in Fig. 2. Interplanner distance and relative intensity are shown in table 1.

Table 1 : X-Ray Powder Diffraction Pattern of Scopolamine Hydrobromide

d(A°)	I/Io%	d(A°)	I/Io%
11.45	92.9	3.04	11.8
9.57	13.8	2.98	27.4
7.73	16.7	2.91	11.2
6.99	9.8	2.86	11.0
6.16	36.1	2.76	36.5
5.81	61.9	2.66	21.6
5.29	74.9	2.59	20.0
5.01	17.4	2.52	13.9
4.76	100	2.47	21.2
4.51	49.9	2.37	22.4
4.31	63.1	2.33	12.4
4.18	55.0	2.27	16.9
4.14	43.0	2.20	10.0
3.99	55.5	2.13	10.6
3.88	10.5	2.07	10.1
3.62	24.1	2.03	10.0
3.58	54.4	2.01	12.8
3.45	36.0	1.99	11.4
3.38	17.0	1.78	10.3
3.30	30.8		
3.15	50.6		
3.12	24.9		

d = interplanner distance, I/Io = relative intensity (based on the highest intensity of 100).

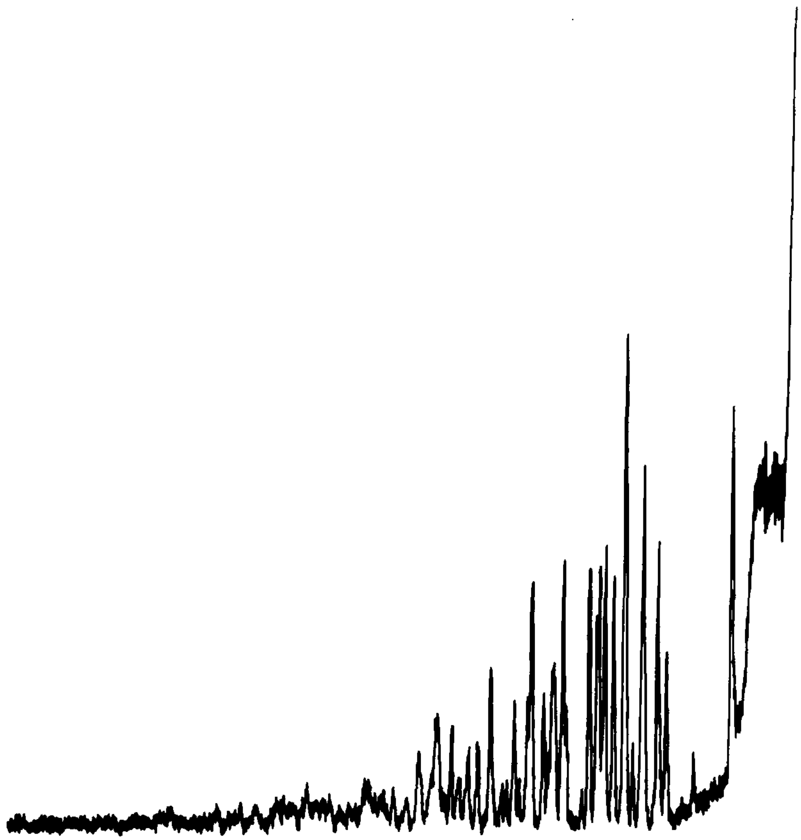


Fig. 2 : X-Ray Diffraction Pattern of Scopolamine Hydrobromide.

2.9 Spectral Properties

2.9.1 Ultraviolet Spectrum (UV)

The UV absorbance spectrum of scopolamine hydrobromide in methanol was scanned from 200 to 400 nm using a Pye-Unicum SP 8-100 spectrophotometer (Fig. 3). Scopolamine hydrobromide exhibited the following absorptivity values (Table 2).

Table 2 : UV absorptivity values

max nm	ε	A(1%, 1Cm)
246	134.5	3.5
252	153.7	4.0
258	172.9	4.5
264	115.3	3.0

Other reported UV data for scopolamine and salts:-

Substance	Solvent	max nm	Ref.
Scopolamine	0.1N H_2SO_4	251 (E1%, 1cm 12) 257 (" " 14) 263.5 (" " 11.2)	(17)
Scopolamine hydrochloride	water	247 (" " 3) 252 (" " 4) 258 (" " 4) 265 (" " 3)	(17)
Scopolamine N-butylbromide	aqueous acid	252 (A_1^1 3.7) 258 (" 4.6) 264 (" 3.6)	(16)

2.9.2 Infrared Spectrum (IR)

The IR spectrum of scopolamine hydrobromide as K Br disc (1:200) was recorded on a Perkin Elmer 580B Infrared spectrophotometer (Fig.4). Assignment of the functional groups have been correlated with the following frequencies (Table 3).

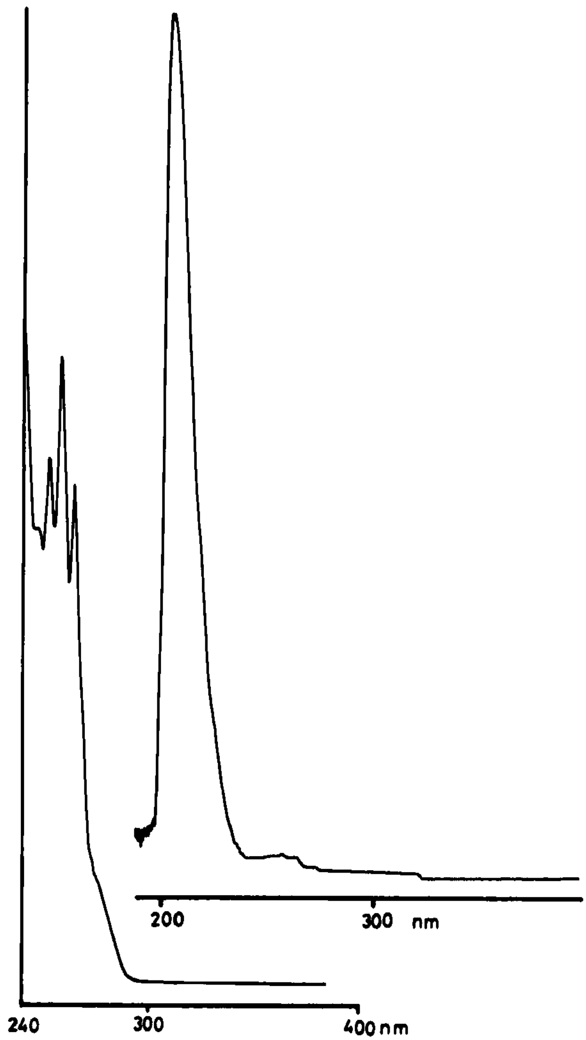


Fig. 3 : UV Spectrum of Scopolamine HBr (MeOH)

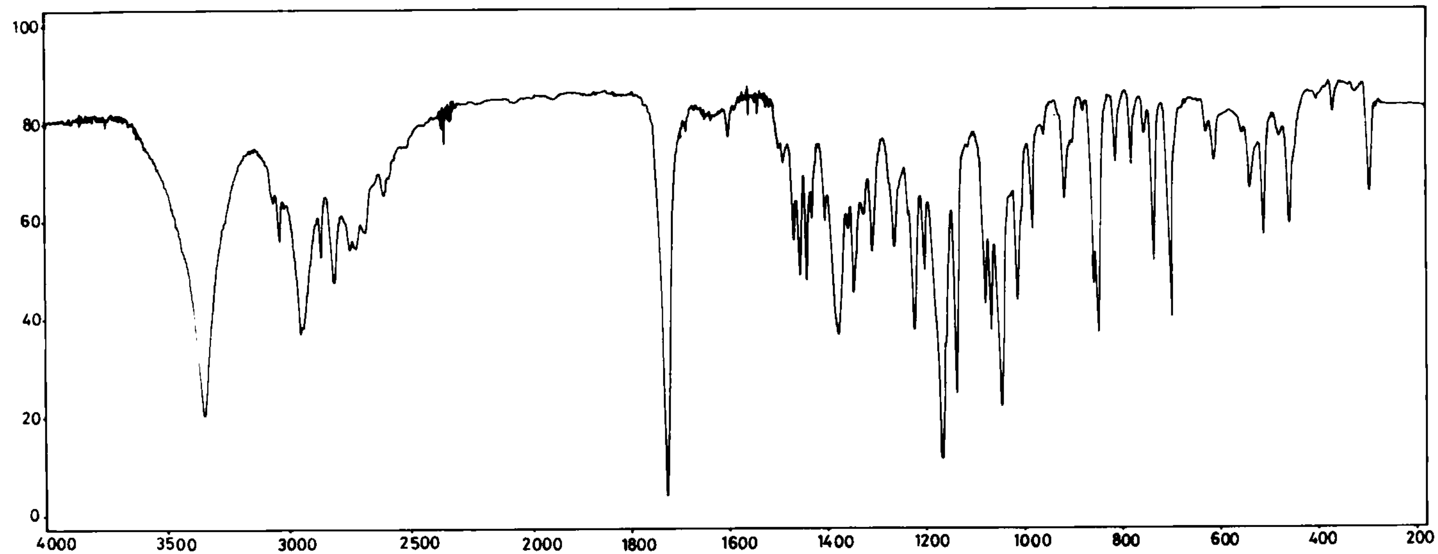


Fig. 4 : IR Spectrum of Scopolamine Hydrobromide (KBr-Pellet).

Table 3 : IR Characteristics of Scopolamine

Frequency Cm^{-1}	Functional group
3350(s)	OH (hydrogen bonded)
2950	CH (stretch)
2810	$N\text{-}CH_3$
1728(s)	$O\text{-}\overset{\overset{O}{\Vert}}{C}\text{-}$(ester)
1600	C=C (aromatic)
1166(s), 1045(s)	C-O-C (ether)
780,735,700(s)	5H (monosubstituted aromatics)

s = strong absorption

The IR exhibited the following other absorption bands:-

1470, 1455, 1440, 1378(s), 1345, 1308, 1262, 1222(s), 1200, 1138(s), 1078, 1068, 1015, 980, 915, 858, 850, 610 cm^{-1}.

Clarke (17) reported the following principal peaks for scopolamine: 1725, 1165, 1060, 1041 cm^{-1}.

For scopolamine hydrobromide in K Br-disc the following principal peaks were reported (16): 1730, 1166, 1047, 853, 736 and 705 cm^{-1}.

2.9.3 Nuclear Magnetic Resonance

2.9.3.1 ^{1}H-NMR Spectra

The proton spectra of scopolamine hydrobromide were recorded, once in D_2O on a Varian FT80A (80 MH_z) NMR spectrophotometer (Fig. 5), and another in D_2O using a Varian XL 200 (200 MH_z) spectrophotometer (Fig. 6) using 4,4-dimethyl-4-silapentane sulfonic acid (DSS) as an internal reference with both.
The proton chemical shifts are assigned and shown in table 4.

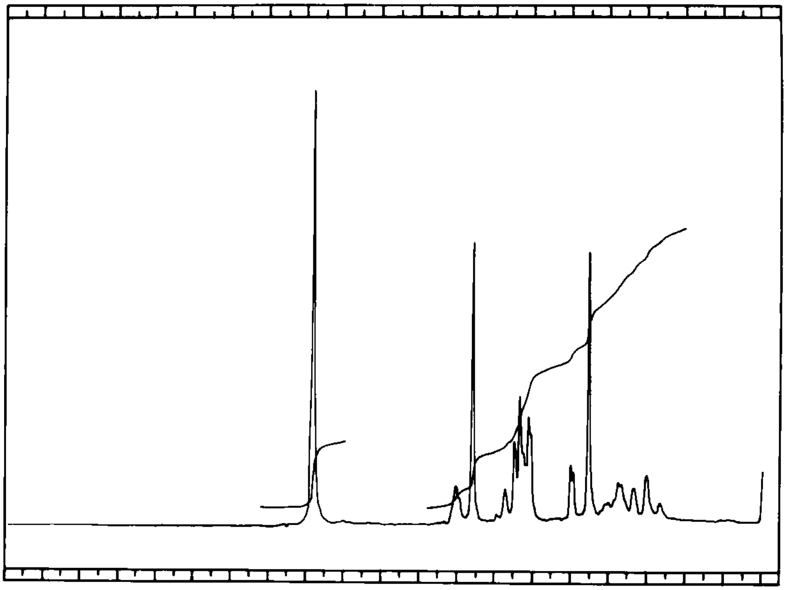


Fig. 5 : ^{1}H-NMR Spectrum of Scopolamine HBr (80 MHz).

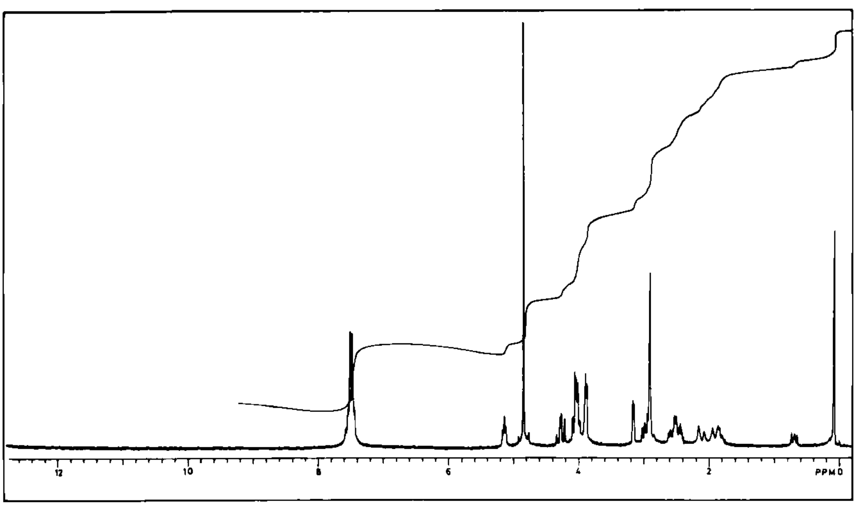


Fig. 6 : ^{1}H-NMR Spectrum of Scopolamine HBr (200 MHz).

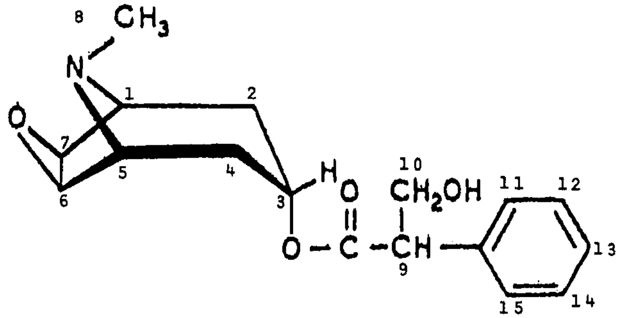


Table 4: ^{1}H-NMR Characteristics of Scopolamine

Proton assignment	Chemical shifts δ (ppm) 80 MHz	200 MHz
5 H aromatics (at $C_{11,12,13,14,15}$)	7.41(s)	7.42,7.47(d)
H at C_3	5.05(t)	5.17(t)
H_2OH at C_{10}	4.75(s)	4.81(s)
H at C_9	4.07(m)	4.02(m)
H at C_1 & H at C_5	3.84(d)	3.88(d)
H at C_6 & H at C_7	3.12(d)	3.13(d)
$N-CH_3(C_8)$	2.84(s)	2.88(s)
2 H at C_2 & 2H at C_4	1.90-2.37 (m)	1.88-2.24 (m)

s = singlet, d = doublet, t = triplet,
m = multiplet

Other ^{1}H-NMR data for scopolamine were also reported (18 -20).

2.9.3.2 Carbon-13 NMR Spectrum

The ^{13}C-NMR spectrum of scopolamine hydrobromide in D2O was recorded on a Varian XL-200 NMR spectrometer, using 4,4-dimethyl-4-silapentane sulfonic acid (DSS) as an internal reference. The spectrum is shown in Fig.7. The carbon chemical shifts were assigned and listed in table 5.

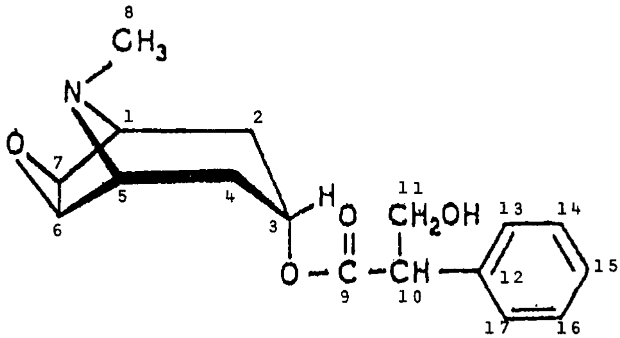


Table 5 : Carbon-13 Chemical Shifts of Scopolamine.

Carbon Assignment	Chemical Shift δ (ppm)	Multiplicity
C_9	172.96	Singlet
C_{12}	136.37	Singlet
C_{13}, C_{17}	129.89	Doublet
C_{15}	129.09	Doublet
C_{14}, C_{16}	128.90	Doublet
C_3	67.36	Doublet
C_{11}	64.35	Triplet
C_1, C_5	62.88	Doublet
C_6, C_7	57.66 57.53	Doublet
C_{10}	54.26	Doublet
C_8	31.18	Quartet
C_2, C_4	24.54 24.50	Triplet

Other ^{13}C-NMR data for scopolamine have been also reported (21-23).

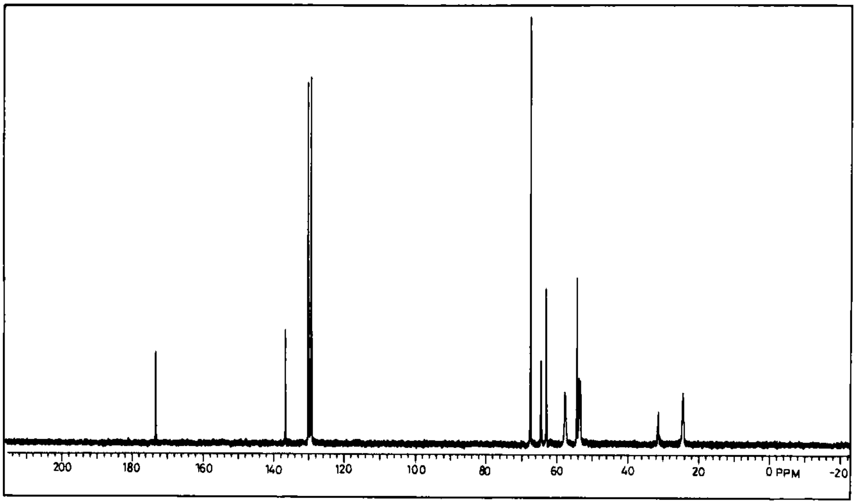


Fig. 7 : Carbon-13 NMR Spectrum of Scopolamine HBr.

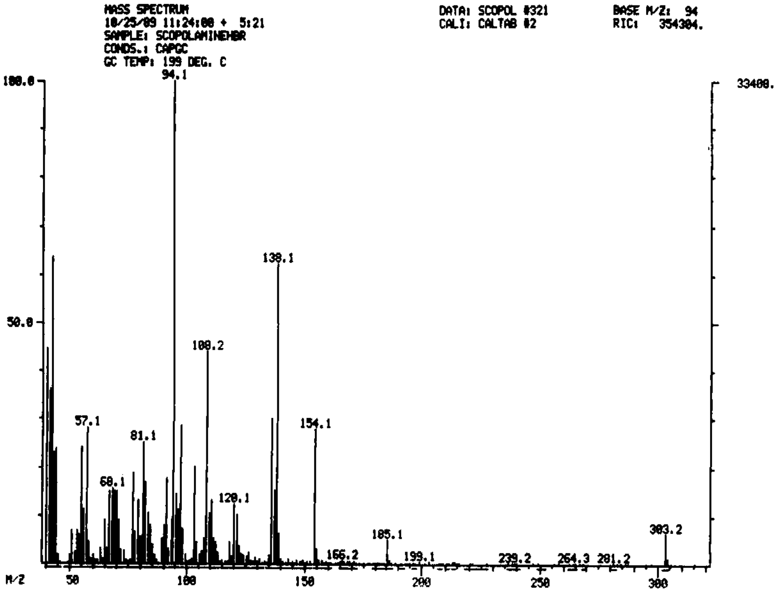
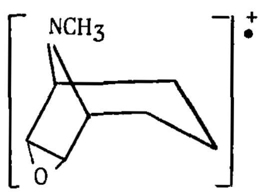
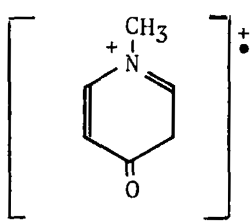


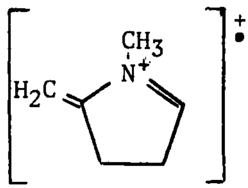
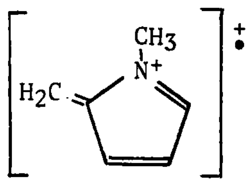
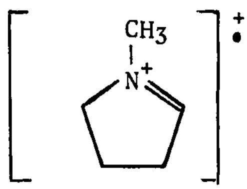
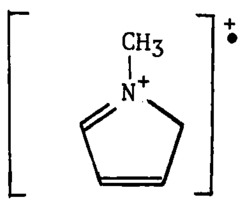
Fig. 8 : Mass Spectrum of Scopolamine.

2.9.4 Mass Spectrum

The electron-impact ionization (EI) mass spectrum of scopolamine HBr is presented in Fig. 8.
The spectrum was obtained using a Finnigan MAT 5100 series GC/MS spectrometer operating with an ionization potential of 70 eV.
The spectrum exhibited a molecular ion peak at a mass/charge (m/z) ratio of 303.2 with relative intensity of 6.4% and a base peak at m/z ratio of 94.1.
The most prominent ions, their relative intensities as well as some proposed ion fragments are shown in table 6.

Table 6 : Mass Fragments of Scopolamine

m/z	Relative % Intensity	Ions
303	6.4	M^{+}
185	5.1	-
154	27.9	–
139	6.4	
138	62.0	[139-H]
137	15.2	[138-H]
136	29.8	[137-H]
121	10.8	—
120	12.7	[121-H]
110	13.3	

m/z	Relative % Intensity	Ions
109	11.4	[110-H]
108	44.3	[109-H]
103	20.3	—
98	28.5	—
97	11.4	[98-H]
96	14.6	
94	100	
91	17.7	—
83	11.4	
82	17.1	
81	25.3	[82-H]
79	13.3	—

m/z	Relative % Intensity	Ions
77	19.0	—
71	8.9	—
70	15.8	[71-H]
69	15.8	[70-H]
68	15.8	[69-H]
67	15.8	[68-H]
65	9.5	—
57	28.5	-
56	11.4	[57-H]
55	24.0	$[CH_2{=}\overset{+}{N}{=}CH - CH_2]^{+\bullet}$
44	24.0	—
43	23.4	[44-H]
42	63.3	$[CH_2{=}N{=}CH_2]^{+\bullet}$
41	36.7	[42-H]
40	44.3	[41-H]

Other mass spectral data of scopolamine have been reported (24-27).

The chemical ionization (CI) mass spectra of scopolamine have been reported (25, 27). Scopolamine exhibited the following prominent ions with their relative intensities.

m/z	Relative % Intensity	Ions
304	(4)	$M{\cdot}H^+$
306	(21)	-
138	(100)	base peak
156	(6)	-

3. Isolation of Scopolamine

Scopolamine occurs along with hyoscyamine and/or atropine in several Solanaceous plants, such as species of *Atropa, Datura, Hyoscyamus, Duboisia, Mandragora* and *Scopolia* (28). In some of these plants *(Atropa* and *Scopolia)*, hyoscyamine is the dominant alkaloid throughout the life cycle of the plant. In *Datura stramonium*, hyoscyamine is the principal alkaloid at the time of flowering and after. Whereas young plants contain principally scopolamine. In many other species of *Datura* (e.g. *D. ferox; D. metel; D. meteloides)*, scopolamine is the principle alkaloid of the leaves at all times (28) and these species are used to isolate scopolamine.

One of the methods for the isolation of scopolamine is described as follows (29):-
The powdered Datura species is throughly moistened with an aqueous solution of sodium carbonate and extracted with ether or benzene. The alkaloidal bases are extracted from the solvent with dilute acetic acid, the acid solution is then shaken with ether as long as the latter takes up coloring matters. The alkaloids are precipitated with sodium carbonate, filtered off, washed and dried.
The dried precipitate is dissolved in ether or acetone, the solution is dehydrated with anhydrous sodium sulfate and filtered. The filtrate is concentrated, cooled when crude hyoscyamine and atropine crystalize from solution. The crystals are collected by filtration and to the mother liquor dilute hydrobromic acid is added to give scopolamine hydrobromide as crystals, which is collected, washed, dried and recrystallized to give pure crystals.

4. Synthesis of Scopolamine

4.1 Partial Synthesis

Since scopolamine is an ester of the aminoalcohol *scopine* and *tropic acid*, it can therefore be obtained by heating scopine with tropic acid in the presence of hydrogen chloride as the same manner of atropine (30).

The esterification can be also performed by treating scopine hydrochloride with acetyltropoyl chloride in nitrobenzene (1).

4.2 Total Synthesis

Several schemes for the total synthesis of scopolamine have been reported. Fodor et al in 1956 (1) *has accomplished the first total synthesis of this alkaloid.*

Scheme I : Fodor's first total synthesis:
Acetyltropenol is an important intermediate in this synthesis, it can be obtained by two alternative routes (31):

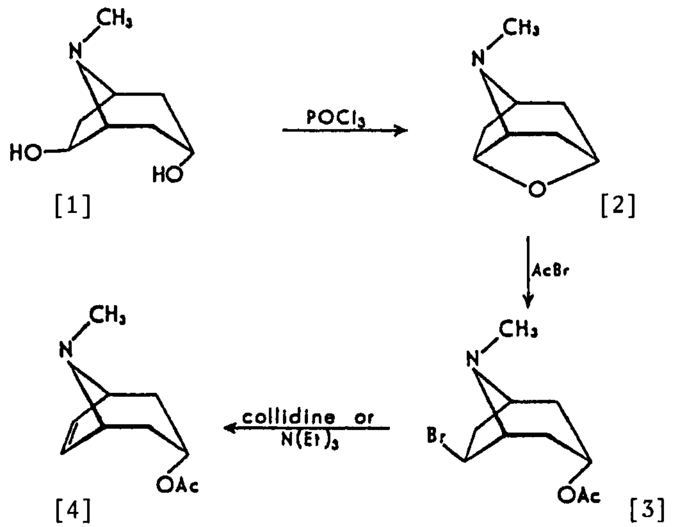
1) From (±) 3α, 6β-dihydroxytropane [1] which is dehydrated to give 3α, 6α-oxidotropane [2], and this on acetobromolysis gives rise to 3α-acetoxy-6β-bromotropane [3]. Dehydrobromination of [3] affords acetyl -6-tropene-3α-ol [4], which on hydrogenation gives acetyltropan-3α ol.
2) By starting with 6-hydroxytropan-3 one phenylurethane [5] which undergoes catalytic hydrogenation over Raney nickel to produce tropan-3α,6β-diol monophenylurethane [6]. This could be acylated easily either with acetyl chloride or with isovaleryl chloride. Distillation in a vacuum of these mixed esters proved sufficient to afford cleavage of the phenylcarbamyl group into phenyl isocyanate and the corresponding 3α-acetoxy-6β-hydroxytropane [7]. The latter gives with *p*-toluene sulfonyl chloride the tosyl-ester [8], which undergoes, in turn, elimination on action of collidine or triethylamine, affording acetyl-6-tropene-3α -ol [4].

[4] is subjected to oxidation with trifluoroperacetic acid thus producing acetylscopine [9]. Hydrolysis of [9] with N NaOH in acetone leads to scopine [10], the hydrochloride of which is acylated, in turn, with acetyltropoyl chloride in nitrobenzene to furnish scopolamine [11] besides a number of by-products. Separation of these can be achieved on a cellulose powder chromatogram and using butanol-N HCl as the eluting solvent, pure scopolamine is resulted (identical with the natural product).

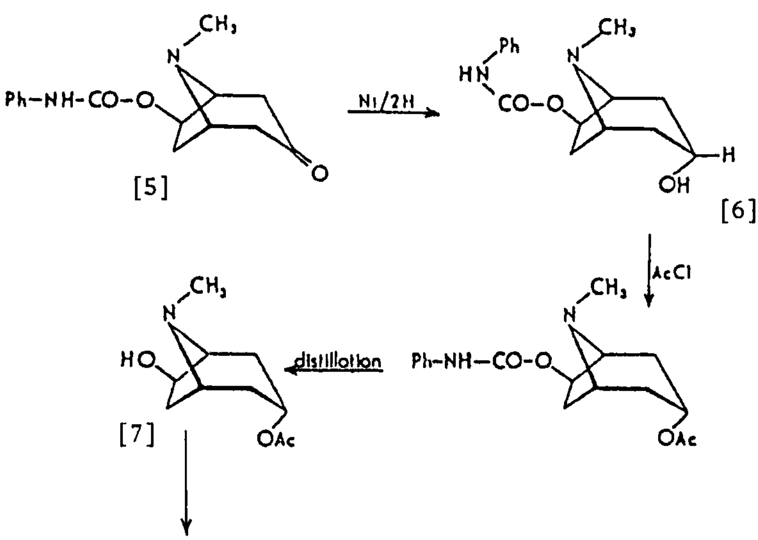
This first total synthesis is presented in scheme I [after 31].

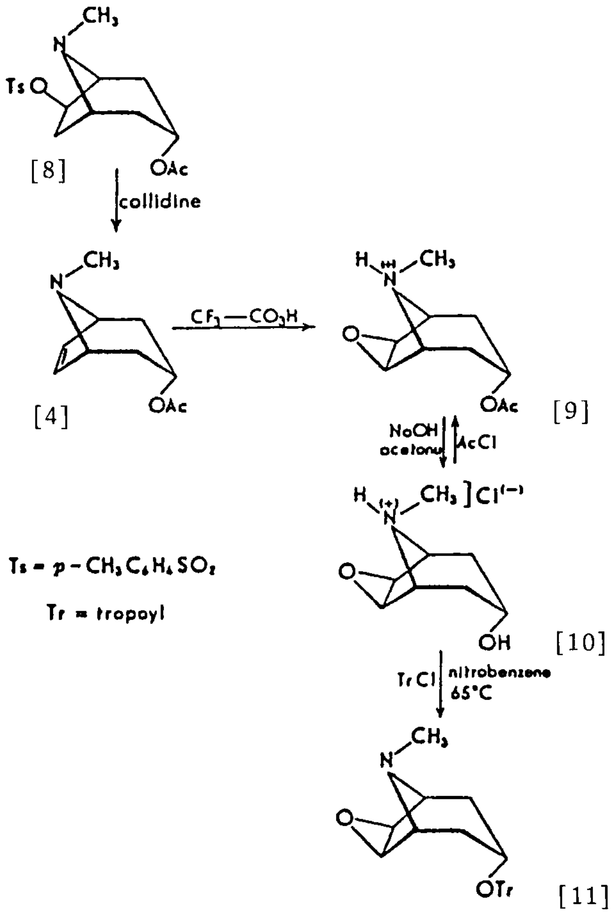
An improved and simplified total synthesis of (-)-scopolamine has been accomplished (2 *), this constitutes the second total synthesis of scopolamine:-* Methoxysuccindialdehyde [2] after liberation from 2,3,5-trimethoxytetrahydrofuran [1] (available from furan) is condensed with methylamine and acetone dicarboxylic acid to give 6-methoxytropinone [3]. This is reduced

Scheme I: The First Total Synthesis of Scopolamine



The alternative route for [4]




[8]
collidine
[4]
CF_3-CO_3H
[9]
NaOH acetone
AcCl
[10]
TrCl
nitrobenzene 65°C
[11]
Ts = p-$CH_3C_6H_4SO_2$
Tr = tropoyl

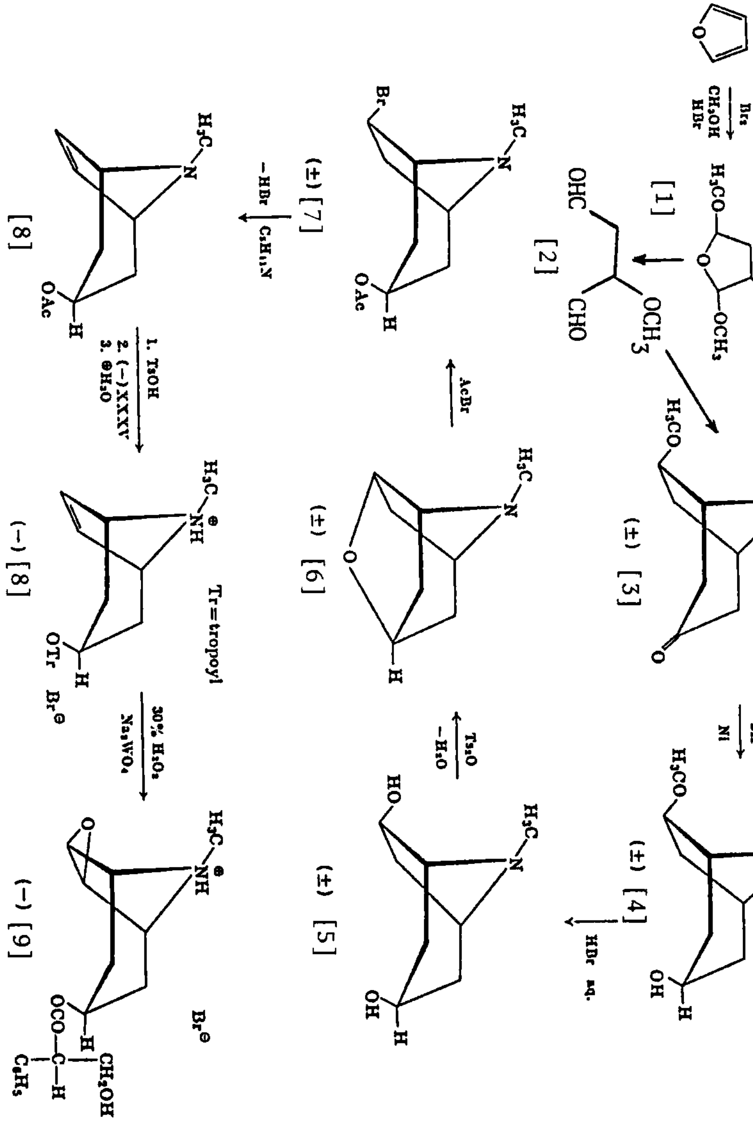
to 6-methoxytropan-3α-ol [4] which is de-O-methylated with aqueous hydrobromic acid to afford (±)-tropan-3α, 6β-diol [5]. Internal cyclization with *p*-toluenesulfonic acid anhydride gives 3α, 6α-oxidotropane [6]. [6] upon cleavage with acetylbromide (acetobromolysis) gives 6β-bromo-3α-acetoxytropane [7]. Piperidine is used to eliminate hydrogen bromide and the ester exchanged in a two-step process to produce 6,7-dehydrohyoscyamine [8]. Epoxidation of the hydrobromide of [8] with 30% hydrogen peroxide in the presence of sodium tungstate as catalyst leads to scopolamine hydrobromide [9].

This improved total synthesis is presented in scheme II [after (2)].

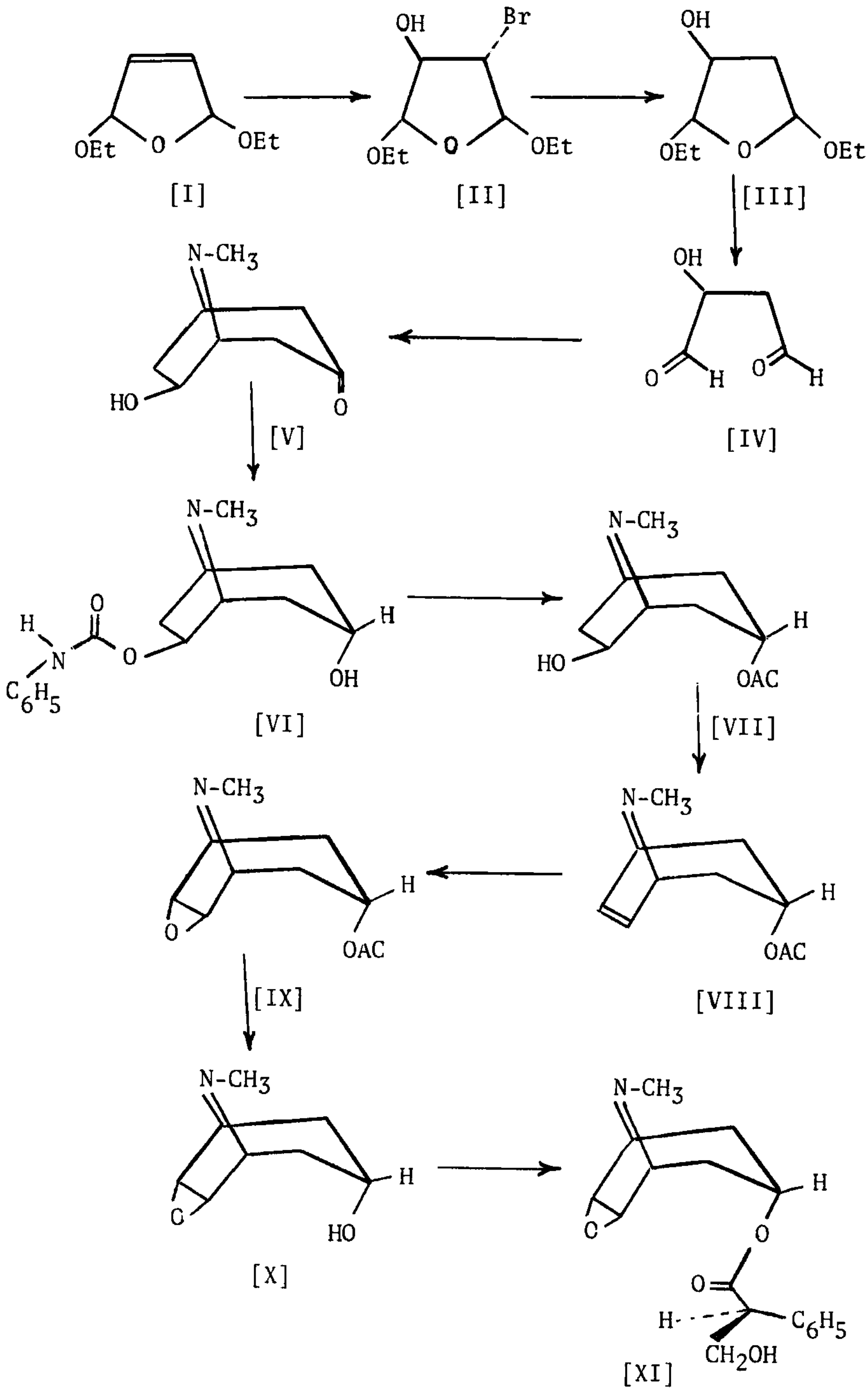
A further modification of the total synthesis of scopolamine has been reported (3 *) though this modification depends on the same principles of the previous two syntheses:-*
2,5-Diethoxy-2,5-dihydropyran [I] (made by anodic oxidation of furan in ethanol) is converted to its bromohydrin [II] and this on basic hydrogenolysis yields 2,5-diethoxy-3-hydroxytetrahydropyran [III]. Mild acid hydrolysis of [III] leads to malic dialdehyde [IV] which undergoes Robinson-Schopf condensation to produce (±)-6-hydroxytropinone [V]. Treatment of [V] with phenylisocyanate leads to a urethane derivative capable of catalytic reduction to tropan -3α,6β-diol monophenylurethane [VI]. Acylation of the latter, followed by distillation results in cleavage of the phenylcarbamyl moiety to phenylisocyanate and the formation of 3α-acetoxy 6β-hydroxytropane [VII]. This is converted into its corresponding tosyl ester with *p*-toluenesulfonyl chloride, elemination of toluenesulfonic acid could be induced with triethylamine to generate acetyl-6-tropene 3α-ol [VIII]. Epoxidation of the trifluoroacetate salt of [VIII] with trifluoroperacetic acid leads to acetylscopine [IX], this upon gentle basic hydrolysis (N NaOH in acetone) affords scopine [X]. Treatment of [X] with (±)-acetyltropoyl chloride followed by hydrolysis with dilute hydrochloric acid produces (±)-scopolamine [XI]. Resolution of [XI] with (+)-tartaric acid leads to (-)-scopolamine.

This modified total synthesis of scopolamine is shown in scheme III.

Scheme II : The Second Total Synthesis of Scopolamine



Scheme III: Modified Total Synthesis of Scopolamine



A different approach to the synthesis of scopine and hence to scopolamine has been recently reported (4):
When a mixture of tetrabromoacetone [I], 1-carbomethoxypyrrole [II] and diironennea (3:1:1.5 mol ration) in benzene is heated at 50° for 72 hours under nitrogen atmosphere, a mixture of the dibromotropenone adducts [IIIa, IIIb] in a 2:1 ratio is obtained. Zinc-copper reduction of the adducts in methanol containing 5% ammonium chloride at 25° for 10 minutes, affords the tropenone adduct [IV]. Reduction of [IV] with diisobutylaluminum hydride (DIBAH) for 23 hours at -78° then for 8 hours at 25° gives rise to the alcohols [Va and Vb]. These can be purified by chromatographic column and converted to scopine [VI] and then into scopolamine.

This newer approach is presented in scheme IV.

4.3 Synthesis of Tropic acid

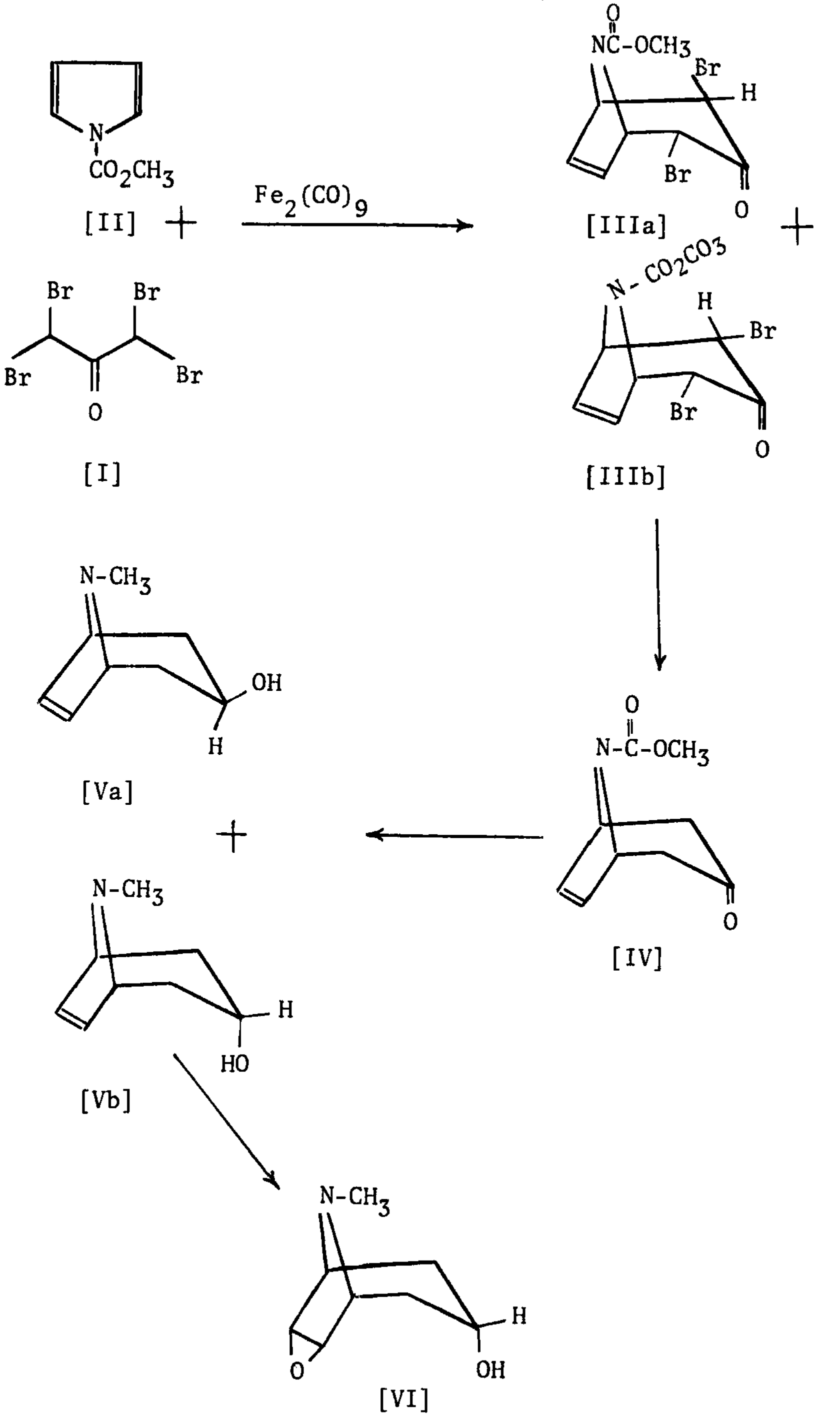
Several schemes for the total synthesis of tropic acid are known (Scheme I to IV).

Scheme I: Landenburg's synthesis (32).
Acetophenone [1] is converted into α, α -dichloroethylbenzene [2] by the action of phosphorous pentachloride. [2] is reacted with potassium cyanide to furnish α-ethoxy-α-cyanoethylbenzene [3]. This is hydrolysed with barium hydroxide solution to give atrolactic ethylether [4]. The latter is heated with hydrogen chloride to yield atropic acid [5] which is converted to tropic acid [6].

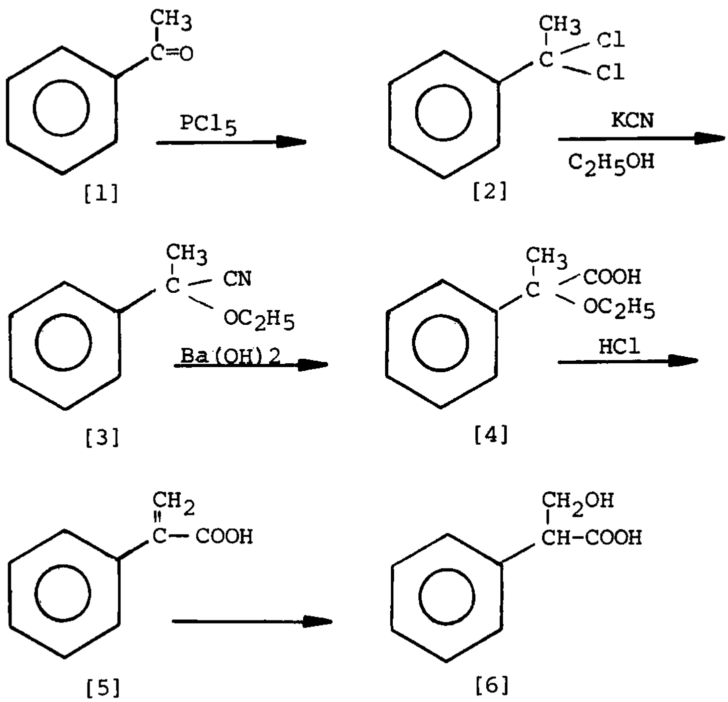
Scheme II: McKenzie and Wood's synthesis (33).
Acetophenone [1] is converted by the action of potassium cyanide to acetophenone cyanohydrine [2]. This upon hydrolysis is converted into atrolactic acid [3]. The latter is heated under pressure to yield atropic acid [4]. Atropic acid [4] is treated with hydrogen chloride in ethereal solution to form β-chlorohydratropic acid [5]. This upon boiling with aqueous sodium carbonate is changed to tropic acid [6].

Scheme III: Müller's synthesis (34).
Ethylphenyl acetate [1] is condensed with ethylformate to give ethyl α-formyl acetate [2]. This on reduction with aluminium amalgam yields dl-tropic ester [3] which upon hydrolysis gives tropic acid [4].

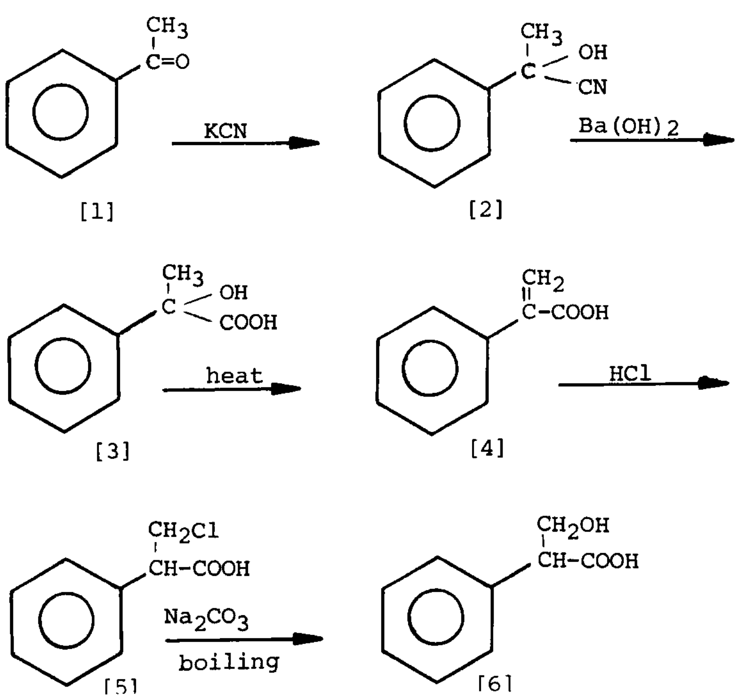
Scheme IV: Recent Synthesis of Scopolamine



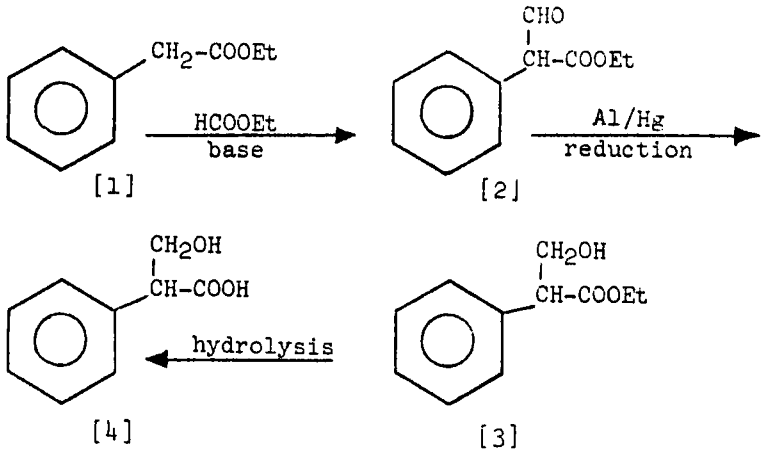
Scheme I: Landenburg's synthesis



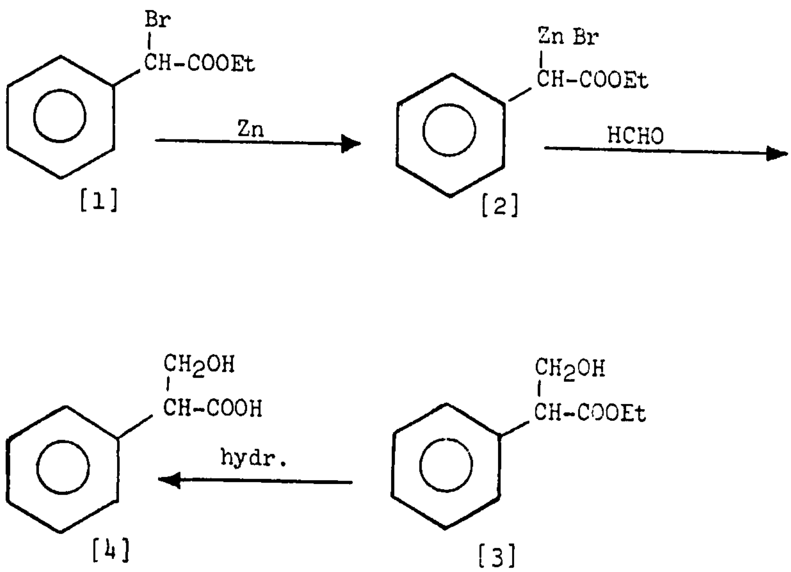
Scheme II: McKenzie and Wood's synthesis



Scheme III: Müller's synthesis



Scheme IV: Chambon's synthesis



Scheme IV: Chambon's synthesis (35).
Ethyl α-bromophenylacetate [1] is treated with Zn to give ethyl-α-zincbromophenylacetate [2] which is treated with formic acid to give dl-tropic ester [3] which upon hydrolysis yields tropic acid [4].

Methods of synthesis of tropic acid are presented in schemes I to IV.

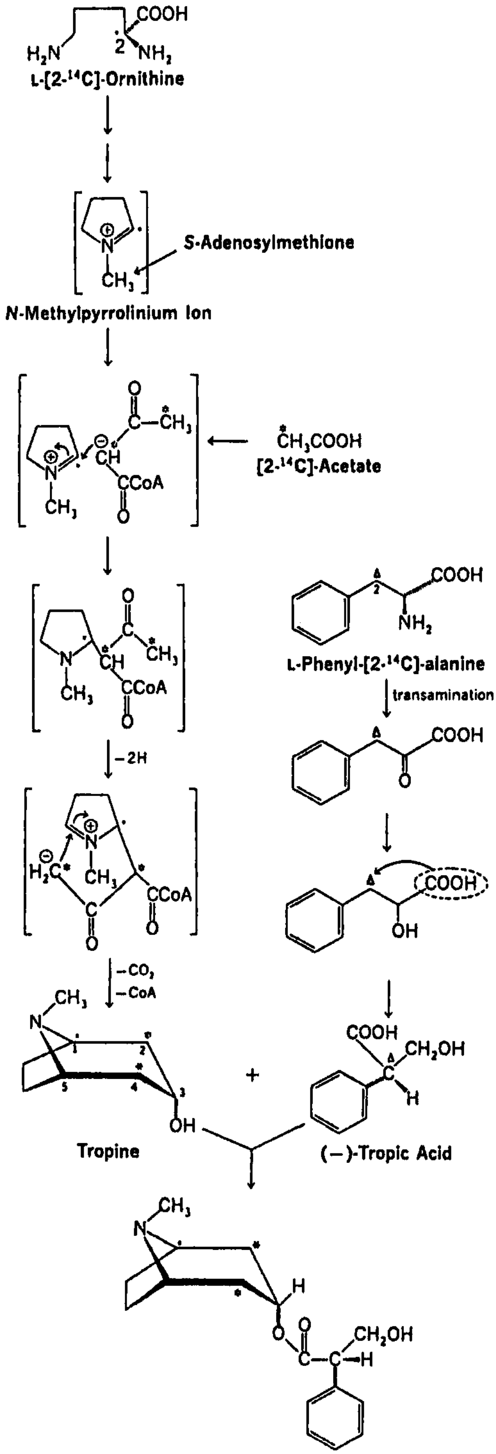
5. Biosynthesis of Scopolamine

Early work with isotopes has established that ornithine (or one of the related aminoacids i.e. glutamic acid or proline (36) and acetate (37,38) are precursors of the tropine moiety (as [2-^{14}C] ornithine; [5-^{14}C] proline and [1-^{14}C] acetate were all incorporated into hyoscyamine isolated from fed plants). It is believed that the incorporation of glutamic acid or proline is considered to occur via ornithine (36), and the acetate is incorporated via acetoacetic acid (36), as feeding with [1,3-^{14}C] acetoacetate resulted in the isolation of radioactive hyoscyamine.
It has been shown that putrescine was incorporated into hyoscyamine (39,40) however, it is predicted that certain enzymes are capable of converting putrescine into N-methylputrescine which it is an established precursor of tropine (36).
It has also been shown that (+)-(*2R*) hygrine serves as a precursor of the tropane alkaloids of *Datura innoxia* (41), this is true as (*2R*)-hygrine is formed by an attack of the acetoacetate on the pyrrolinium salt (36) as it is evident from scheme I.

Very recently it has been reported that the biosynthetic pathway between ornithine and hyoscyamine must be different in *Datura* and *Hyoscyamus albus* (42), i.e. the conversion of ornithine to the 1-methyl-Δ^1-pyrrolinium salt (a precursor of hyoscyamine) can proceed by two pathways, one of which *(in Datura)* can not involve free putrescine.

Phenylalanine is the established precursor of tropic acid. Tracer studies have shown that the side chain of the aminoacid undergoes intramolecular rearrangement during the conversion to tropic acid (43), thus upon feeding L-phenyl-[2-^{14}C] alanine, radioactive (-)-tropic acid is resulted as it is evident from scheme I. Esterification of tropic acid with tropine produces hyoscyamine.

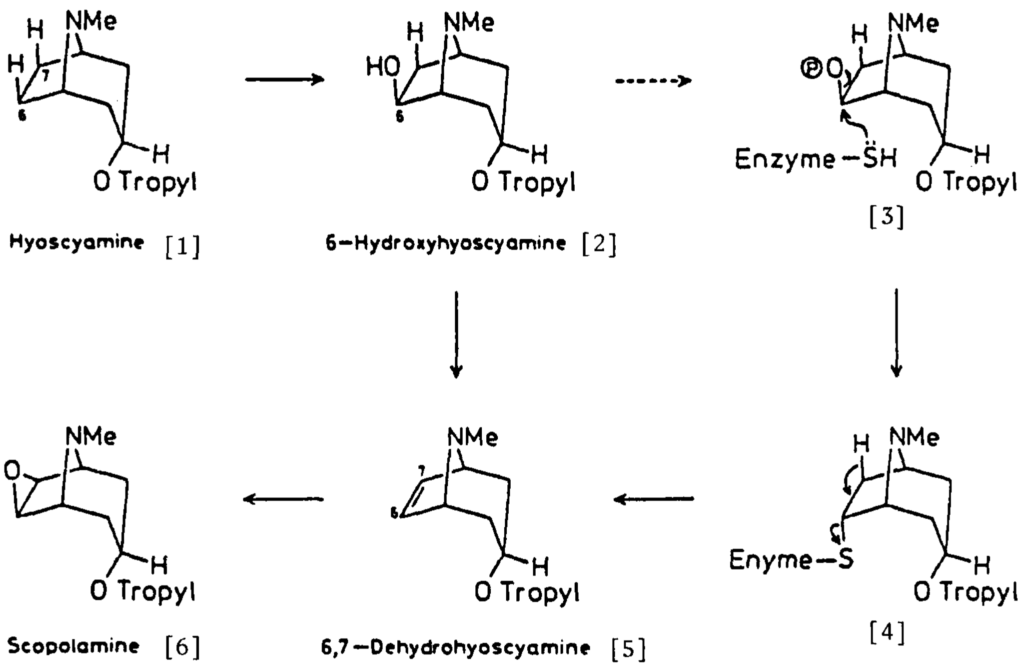
The biosynthesis of hyoscyamine is presented in scheme I [after (44)].



Scheme I:The Biosynthesis of (−)-Hyoscyamine

Scopolamine appears to be formed in the plants from hyoscyamine (45,46,47). It is believed that hyoscyamine [1] is metabolized in the plant to 6-hydroxyscopolamine [2], and this is further metabolized into 6,7-dehydrohyoscyamine [5]. Epoxidation in vivo of [5] gives rise to scopolamine [6]. Leete however, suggested that the dehydration takes place by a two step mechanism. A phosphorylated 6-hydroxyhyoscyamine [3] could be attacked by a nucleophilic reagent (represented by the SH-group of an enzyme) to afford [4]. The double bond of [4] is then generated by a *trans* - elimination to give [5].

The biosynthesis of scopolamine from hyoscyamine is presented in scheme II [after Leete (36)].



Scheme II : Biosynthesis of scopolamine from hyoscyamine.

6. Pharmacokinetics

6.1 Drug Absorption

Scopolamine is rapidly absorbed from the gastrointestinal tract following oral doses of the hydrobromide (17,48,49).It also enters the circulation when applied locally to the mucosal surfaces of the body (48). Only limited absorption occurs from the intact skin (48).
The quaternary derivatives of scopolamine, such as the N-butylbromide or the methobromide are poorly absorbed from the gastrointestinal tract (49) and do not readily pass the blood-brain barrier (49). The total absorption of quaternary ammonium derivative of the belladonna alkaloids, after an oral dose is only 10 to 20% (50,51).

6.2 Onset and Duration

- Following instillation of 1 drop of 0.5% solution, maximum mydriasis occurs within 20 to 30 minutes; mydriasis is observed for up to 7 days (3 to 7 days) (48).
- Following single IM doses of 0.05 to 0.4 mg scopolamine, peak decreases in pulse rate were evident at 1 to 2 hours; decreases in pulse rate persisted for approximately 8 hours (52).
- Following single IM doses of 0.05 to 0.4 mg scopolamine, peak decreases in saliva flow were evident at 1 to 2 hours; this salivary flow was reduced for approximately 8 hours (52).

6.3 Distribution

Scopolamine HBr is bound to plasma proteins (17). Following a single oral dose equivalent to 415 μg of scopolamine to 10 subjects, a mean peak plasma concentration of 0.0003 μg/ml was attained in 0.5 to 1 hour, decreasing to 50% of the peak concentration in 2 to 4 hours (53).
Scopolamine crosses the blood-brain barrier and it has been stated to cross the placenta (49).

6.4 Excretion

- Only about 1% of an oral dose of scopolamine is eliminated as such (unchanged) in the urine (48).

- In rabbits, 2% of an oral dose and 30% of an intravenous (IV) dose are excreted in 3 days (17).
- About 5% of an oral dose is excreted in the urine as unchanged drug (16).
- Urinary excretion of scopolamine was determined using a radioligand binding assay in 9 healthy subjects following administration of approximately 450 µg of the drug over 64 hours via a transdermal delivery system. During the time of medication and 48 hours thereafter, a mean total of 156 µg of scopolamine is excreted in the urine of which 21% was unchanged drug and the remainder 79% metabolized mainly as glucuronide and/or sulfate conjugates. Steady state urinary concentrations were achieved about 12 hours after applying the medication. Concentrations in plasma were below the detectable level of 1.2 ng per ml (49,54).
- Following a subcutaneous injection of 190 mg of scopolamine N-butylbromide in 3 divided doses over 4 hours, the urine collected contained the equivalent of 26 mg of the drug (55).
- Scopolamine is excreted in minimal amounts in breast milk (if at all) and the drug can be administered to nursing mothers. It does not significantly affect milk secretion (56).
- About 90% of an oral dose of scopolamine butylbromide is eliminated in the faeces and less than 10% is excreted in the urine (16). After IV administration of this salt, about 40% of the dose is excreted in the urine (16).

6.5 Metabolites

As scopolamine is an ester alkaloid, it is possible that small amounts are hydrolysed in the serum (57) giving rise to *tropic acid* as well as *scopine or scopoline.*
Scopolamine glucuronides and sulfates are reported to be the main scopolamine metabolites in man (54, 57), while *scopolamine 9'-glucuronide* is the main metabolite in the mouse (54, 57). Other reported scopolamine metabolites :- *aposcopolamine; norscopolamine; norscopolamine-9'-glucuronide and 6-hydroxy(-)-hyoscyamine (57, 58).*

7. Pharmacology and Therapeutic Uses

This section is written by Dr. Zaki (59).
Scopolamine exerts the following pharmacological effects:

Central:

1) Sedative and tranquilizing effects with euphoria, but in elderly and females, it leads to confusion and excitement.

2) Amnestic action: It leads to twilight sleep with morphine in analgesia during labor.

3) Antiepileptic action in grandmal epilepsy by action on cortical centres.

4) Antikinetic action: to control regidity and tremors in chorea and Parkinsonism to block the central cholinergic system in basal ganglia.

5) Hypnotic action by affecting the brain-stem arousal mechanism which reaches the cerebral cortex.

6) Anti-emetic action: by inhibiting the chemo-receptor trigger zone of the vomiting centre in the medulla.

7) Central respiratory centre stimulation.

Peripheral:

1) It possesses parasympatholytic action similar to atropine but five times more powerful on exocrine secretions e.g. lacrymal, salivary, and bronchial as well as on eye in form of passive mydriasis, cycloplegia and increase intraocular pressure.

2) On cardiovascular system: it produces vasodilation of the blush skin area with slight lowering in blood pressure and tachycardia by decreasing vagal tone on the heart.

3) On gastro-intestinal tract: it decreases tone and amplitude of motility with marked decrease in salivary secretion but less decrease in gastric and pancreatic secretions.

Therapeutic uses:

1) Pre-anesthetic medication: to produce sedation thus shortens stage of induction and antagonizes the respiratory depression of morphine and other depressants. It is also more powerful on glandular secretions eg. bronchial and salivary decreasing respiratory complications.

2) In motion sickness and Manier's attacks.

3) In labor with morphine to induce twilight sleep analgesia.

4) In parkinsonism and chorea to reduce dyskinesia and regidity.

5) Locally as eye drops or ointement to produce mydriasis and cycloplegia in corneal and iris lesions. It is less irritating with rapid recovery especially in patients sensitive to atropine.

Contraindications

Scopolamine should not be administered to patients with asthma, hepatitis, or toxemia of pregnancy (60).

8. Drug Stability and Storage

Scopolamine is readily racemized in the presence of dilute alkali (61).
Scopolamine hydrobromide solutions are incompatible with alkalis, silver salts and tannic acid.(49).
Scopolamine hydrobromide as it is or as tablets should be preserved in tight, light resistant containers (14).
Scopolamine injections are preserved in light resistant single dose or multiple dose containers (14).
Scopolamine hydrobromide ophthalmic solutions should be stored in tight containers at a temperature less than 40°C, preferably between 15-30°C, freezing should be avoided (14, 61).
Scopolamine hydrobromide ointment is preserved in collapsible ophthalmic ointment tubes (14).

9. Methods of Analysis

9.1 Identification

The following identification tests are mentioned in the British Pharmacopoeia (12) under hyoscine hydrobromide.

- To 1 mg add 0.2 ml of fuming nitric acid and evaporate to dryness on a water bath. Dissolve the residue in 2 ml of acetone and add 0.2 ml of a 3% w/v solution of potassium hydroxide in methanol; a violet color is produced.
- Yields the reactions characteristic of alkaloids and the reactions characteristic of bromides.

The following tests are mentioned in the USP (14) under scopolamine hydrobromide.

- Dissolve 3 mg in 1 ml alcohol, and evaporate the solution on a steam bath to dryness. Dissolve the residue in 0.5 ml chloroform, add 200 mg of potassium bromide and 15 ml of ether, and stir frequently during 5 minutes. Decant the solvent, dry the residue on a steam bath until the odor of the solvent no longer is perceptible, and compress the residue to a disc. The infrared absorption spectrum of the resulting potassium bromide dispersion, previously dried at 105° for 3 hours, exhibits maxima only at the same wavelengths as that of a similar preparation of USP Scopolamine Hydrobromide R.S., treated in the same manner.
- To 1 ml of a solution (1 in 20) add a few drops of chlorine TS, and shake the mixture with 1 ml chloroform, the latter assumes a brownish color.
- Add to scopolamine hydrobromide injection silver nitrate TS, a yellowish white precipitate is formed, which is insoluble in nitric acid but slightly soluble in 6 N ammonium hydroxide.

Other identification tests are as follows:-

- Gold chloride test (62).
  To a few ml of a 1% aqueous solution of scopolamine hydrobromide acidified with hydrochloric acid, add a few drops of gold chloride solution; a lemon yellow oily precipitate is formed which crystallizes after a while. The precipitate is then recrystallized from boiling water acidified with dilute HCl and dried, scopolamine produces lustrous yellowish broad prisms; m.p. 208-209°.
- Gerrard reaction (63).
  To a few mg of the alkaloid, a 2% v/w solution of

mercuric chloride in 50% alcohol, a white precipitate is produced.

- *Identification of scopolamine tablets:*
Digest a quantity of the finely powdered tablets equivalent to about 0.005 g of scopolamine hydrobromide with 20 ml of ethanol (95%) for one hour, filter and evaporate the filtrate to dryness on a water-bath. Dissolve the residue in 10 ml water, add 2 ml dilute ammonia, and extract with 3 quantities each of 5 ml of ether. Filter the mixed ethereal extracts into a small glass dish, and carefully evaporate the ether on a water-bath. Dissolve the residue in 1 ml 0.1 N HCl with the aid of gentle heat, add to the solution 0.05 g potassium bromide and 0.5 ml of gold chloride solution (TS). After several hours draw off the liquid and wash the crystals of scopolamine auribromide with small quantities of water. Melting temperature of the crystals after drying over phosphorous pentoxide in vacuo for two hours about 192° (13).

9.2 Microcrystal Tests

A 0.1% w/v of scopolamine HBr in water was prepared for the microcrystal tests. 1 to 2 drops of this solution is treated on a microscopical glass slide with equal drops of the specific reagent, after a while the crystals so formed were microscopically examined (64).

- Kraut's reagent (modified potassium bismuth iodide solution) gave with scopolamine after 5 minutes, rosettes of plates (Fig. 9).
- Gold chloride solution, furnished after 4 minutes curved plates (Fig. 10).
Clarke reported a sensitivity (1:1500) for the first test, and (1:400) for the second test (17).

Microcrystal test can be performed to identify tropane alkaloids after extraction from animal tissues (65):
These alkaloids are extracted from animal tissues with the universal buffer solution or with acid solution at pH 4.0 to 5.0 (oxalic or tartaric acid). The alkaloids are then identified microcrystallographically by reaction with Rieneck's salt and by Vitali reaction (65).

Fig 9 : Microcrystals of Scopolamine with Kraut's Reagent.

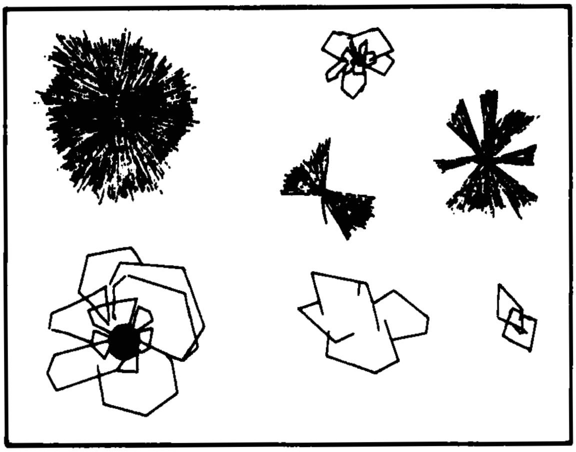
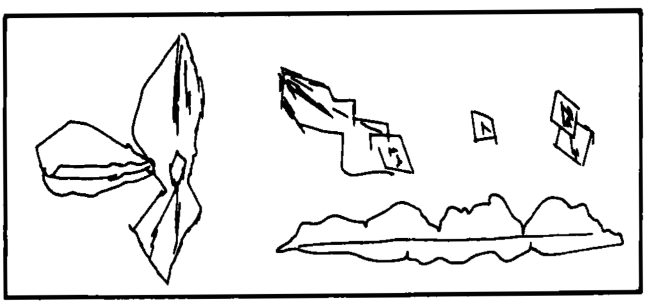


Fig 10 : Microcrystals of Scopolamine with Gold Chloride.



9.3 Titrimetric Determinations

9.3.1 Aqueous Titrations

Scopolamine HBr as salt or as some of its formulations can be assayed by acid-base titration (66):
The scopolamine base is extracted with chloroform from ammonical solution. The combined chloroform extracts is washed, evaporated to dryness and titrated to the end point with 0.05N acid (sulfuric acid or HCl) using methyl red as an indicator *1 ml 0.05N acid = 0.02192g of* $C_{17}H_{21}O_4N$, *HBr*, $3H_2O$.

- Determination of small amounts of nitrogenous bases (including tropine alkaloids) in aqueous solutions such as eye-drops and injections has been described (67).
The method depends on precipitation of the alkaloid with tetraphenylboron at pH 3.7 and the excess of the reagent is then determined by back-titration with a standard solution of quaternary ammonium salt to a visual end point. *Melting points of the organic tetraphenylboron salts may be used in the identification of many of these compounds* (66,67).

Scopolamine butylbromide as well as other quaternary ammonium bases of tropane alkaloids can be estimated by titration after passage of their aqueous solutions through a suitable ion exchange resin as follows (68):
A weighed quantity of the sample is dissolved in 10 ml of water and passed through a resin column packed with IR A 400 at a rate of 1 ml per minute. The column is finally washed with water. 10 ml of 0.1 N HCl and 10 ml of water are added to the eluate and the excess of acid is back titrated with 0.1N NaOH solution.

9.3.2 Non-aqueous titration

The USP (14) describes a non-aqueous titration method for the assay of scopolamine hydrobromide. Dissolve about 750 mg of scopolamine hydrobromide, accurately weighed in a mixture of 30 mL of glacial acetic acid and 10 mL of mercuric chloride TS, warming slightly to

effect solution, cool at room temperature, add 2 drops of crystal violet TS and titrate with 0.1 N perchloric acid VS. Perform a blank determination and make any necessary correction. *Each 1 ml of 0.1 N perchloric acid is equivalent to 38.43 mg of* $C_{17}H_{21}NO_4$. *HBr.*

The BP (12) recommends the following procedure: Dissolve 0.4 g of scopolamine HBr (or 0.3 g scopolamine butylbromide) in 20 ml of anhydrous glacial acetic acid, add 10 ml of mercuric (II) acetate solution TS and 1-naphtholbenzein solution as an indicator. Titrate with 0.1 M perchloric acid VS, determining the end point potentiometrically.
Each ml of 0.1 M perchloric acid VS is equivalent to 0.03843 g of $C_{17}H_{21}NO_4$*,HBr. or is equivalent to 0.04404 g of* $C_{21}H_{30}BrNO_4$ *(Scopolamine butylbromide).*

Small amounts of alkaloids (scopolamine HBr or atropine sulfate) in injection can be estimated in non-aqueous medium by using *toluene-p-sulfonic acid* as follows (69).
Aqueous solution (2 to 5 ml, containing 2 to 5 mg of the alkaloid) is treated with sodium bicarbonate to pH 8 to 9. The alkaloid is extracted with chloroform (15, 10 and 5 ml) and the combined extracts are filtered through filter paper. The filtrate is titrated with 0.005 N *toluene-p-sulfonic acid* in dioxan containing 1% phenol in the presence of dimethyl-yellow as an indicator.
The method is used for the determination of scopolamine hydrobromide (or atropine sulfate) in the presence of morphine hydrochloride:-
The sample is made alkaline with NaOH solution to pH 11 and scopolamine (or atropine) is extracted with chloroform and determined as above.

Many other titrimetric methods for the assay of scopolamine have been published, some of these are in references (70-74).

9.3.3 Potentiometric Titrations

Scopolamine among other alkaloids is easily titrated in aqueous solutions at concentrations of 0.01 to 0.03M using the glass electrode for direct replacement reactions (75).

Potentiometric titration may be carried out by using a glass electrode and a standard calomel cell as reference electrode (12).
Titrate with the titrant to the color change of the indicator that corresponds to the maximum value of dE/dV (where E is the electromotive and V the volume of titrant).

Hydrochlorides of several alkaloids and related substances including scopolamine were titrated in dimethyl sulfoxide medium with 0.1M $AgNO_3$. End points were determined by conductometric, potentiometric and polarimetric techniques and the results were satisfactory by these three techniques (76).

9.4 Polarographic Methods

Several alkaloids including scopolamine as salts (hydrobromide, hydrochloride, nitrate) were determined qualitatively and quantitatively by this method (77).
Concentrations of 0.001% (or lower) to 1% of alkaloids can be performed. Alkaloids were qualitatively separated at the cathode of an electrolytic cell. Of the several kinds of electrodes tested, aluminum as anode (wrapped in parchement) and a steel one as cathode were most satisfactory.
The alkaloids collected at cathode were washed, dried and their melting points were determined for identification. Additional of NaCl to the electrolyzed solution hastened the separation of alkaloids. Some alkaloids were quantitatively determined.

— Ocillopolarographic studies of several alkaloids with tropane and isoquinoline linkages have been reported (77a).
Various related alkaloids can be distinguished even in mixtures by identations in the oscillograms. Thus it was possible to distinguish atropine, hyoscyamine, scopolamine, homatropine HBr, cocaine, hydrastine, berberine as well as other alkaloids.

9.5 Spectrophotometric Methods

9.5.1 Colorimetric Determinations

Morin (78) suggested the use of Vitali's reaction for the determination of small amounts of atropine.
Allport and Wilson (79) have also adopted Vitali's reaction for the rapid determination of the alkaloids in belladonna and stramonium. The method was found not applicable to *Hyoscyamus niger* or its galenical preparations (66). Allport and Jones (80) confirmed that atropine reacts quantitatively the same as hyoscyamine and the method is also applicable to scopolamine.

A summary of this determination can be described as follows:-
A quantity of alkaloidal solution or tablets containing between 1.6 to 2.4 mg of atropine is rendered alkaline and extracted with chloroform. The alkaloid is re-extracted from the chloroform with 6% acetic acid and ethanol. An aliquot of the resulting extract is transferred into an evaporating dish and evaporated just to dryness on a water bath, fuming nitric acid (0.2 ml) is immediately added to the residue and again evaporated to dryness. The resulting residue is dissolved in acetone and made up to volume (10 ml). A 3.0% potassium hydroxide in methanol (0.1 ml) is added and the mixture allowed to stand for 5 minutes. A purple color is developed and the intensity of this color is then measured in a photoelectric absorptiometer. The concentration is calculated from a calibration curve with quantities of 0.025 mg to 0.15 mg of pure hyoscyamine treated similarly.

A newer approach to the vitali type of reaction has been reported by Freeman (81): A measured quantity of alkaloidal solution (containing about 0.05 to 0.15 mg alkaloid) is evaporated to dryness on a water-bath, the residue is nitrated with fuming nitric acid (0.2 to 0.3 ml) and evaporated again to dryness. The residue is transferred to a 10-ml graduated flask with the aid of small quantities of dimethylformamide, 25% w/w aqueous

solution of tetramethylammonium hydroxide (0.3 ml) is added to the flask which diluted to volume with dimethylformamide. The resulting mixture is allowed to stand for 5 minutes and the extinction of the developed color is measured at *540 nm* in 1-cm cells against dimethylformamide. The alkaloidal content is ascertained from a calibration curve which is linear (66,81).

A mixture of atropine and scopolamine were quantitatively separated by electrophoresis and estimated by the same method (82): Atropine and scopolamine were separated with 0.1 N aqueous ammonia as the electrolytes. After elution of each alkaloid, the eluate was evaporated on a water-bath to dryness and nitrated with fuming nitric acid, the residue was dissolved in dimethylformamide and treated as above. The extinction (y) of the produced color was measured at *545 nm* and the concentration (X µg per ml) of each alkaloid was calculated from the following equation:

for atropine $X = 113y + 0.8$
for scopolamine $X = 113y + 1.3$

A colorimetric method for the determination of small amounts of tropic acid, mandelic acid and their esters (atropine, scopolamine and homatropine) has been reported (83). Scopolamine is nitrated for 15 minutes with a solution of 20% KNO_3 in concentrated H_2SO_4. On making the nitrated product alkaline with hot 18-20% NaOH, a color develops in 30 minutes. This color is estimated by using an S_{42}, S_{47} or S_{50} filter in the Pulfrich photometer. The sensitivity is 50 and 60 µg of scopolamine per ml. The probable error is ±3.0%.

Colorimeteric estimation of atropine and related alkaloids (including scopolamine) in pharmaceutical preparations has been reported by two procedures (84).
Procedure (a): An aliquot chloroform extract prepared by the USP method (containing 0.25 to 1.0 mg of alkaloids) was evaporated to dryness on a water bath. Fuming nitric acid (0.3) was added and heated till fumes ceased, then the residue was dried at 105° for 15 minutes and

allowed to cool. The residue was dissolved in acetone and diluted to 25 ml. An aliquot (5 ml) was mixed with isopropylamine (2 ml) and 0.1% methanolic KOH (0.1 ml). The extinction of the produced color at *540 nm* was measured after one minute.

Procedure (b): The residue was nitrated as in procedure (a) and dissolved in 50% ethanol (10 ml). The ethanolic solution was heated on a water bath with 10% HCl (2.5 ml) and zinc dust (0.1 g) for 10 minutes, cooled and filtered. The zinc residue was washed with water and the washings were added to the filtrate. 1% $NaNO_2$ (1 ml) was added, mixed and allowed to stand for 10 minutes. 2.5% solution of ammonium sulfamate (1 ml) was added, the mixture was shaked and allowed to stand for 10 minutes. 1% N-1-naphthylethyl-enediamine dihydrochloride solution (1 ml) was added and diluted to 25 ml with water. The extinction of the produced color was measure after 30 minutes at *550 nm*. The concentration was calculated by reference to a standard curve. Recovery experiments in both procedures indicated an accuracy of ±1%.

A photometric method for the quantitative determination of tropane alkaloids has been described (85).

The determination of scopolamine (or atropine or hyoscyamine) is based on the reaction of the alkaloid with p-dimethylaminobenzaldehyde reagent in concentrated H_2SO_4. The intensity of the color so produced being measured in a photoelectric absorptiometer using a green filter.

This method can be applied for the micro-determination of scopolamine (or atropine) which required special treatment, and measuring the extinction at *500 nm* using a suitable spectrophotometer (86).

A method for the determination of scopolamine hydrobromide or atropine base has been reported (87). In this method, the alkaloid is precipitated with molybdophosphoric acid, the precipitate can be dissolved and reduced to molybdium blue which can be colorimetrically measured. The following procedure was described:

To 1 ml sample (containing 0.2 to 1 mg alkaloid)

add 10% H_2SO_4 (1 drop), 5% NH_4Cl solution (0.2 ml) and 0.5% molybdophosphoric acid (dropwise 1 ml). After 10 minutes, filter the mixture, repeat the filtration, wash the precipitate with 0.15% H_2SO_4 (5x1 ml) and with water (5x1 ml), dissolve the precipitate in acetone (2 ml), add ethanol (2 ml) and 2% ascorbic acid solution (1 ml), set aside for 15 minutes, add acetone (1 ml), cool to 20° and dilute with water to 10 ml. Measure the extinction at *430 nm* (or use an appropriate filter) against water in 2-ml cells. Beer's law is obeyed with stated concentrations. The error is < 3.0% (87).

N-butylscopolamine bromide as well as other alkaloids can be assayed as follows (88).
Scopolamine salt (< 0.5 mg) is extracted from paper chromatography with citrate-phosphate buffer solution (pH 7.5) (20 ml), 0.15% bromothymol blue solution (1 ml) is added and the mixture extracted with chloroform (3x20 ml). The combined chloroform extracts are mixed with 2% ethanolic H_3BO_3 (25 ml), filtered and the resulting filtrate is diluted to 100 ml with ethanol. The extinction is measured at *436 nm* against a reagent blank in 2-cm cells. The reproducibility ≅ ± 2%.

The colorimetric reaction of Tropaeolin 00 with alkaloids under acidic conditions is used for microquantitation of alkaloids (Haussler 89).
The aqueous solution of an alkaloid (5 ml) (containing ≅ 100 μg) is mixed with a similar quantity of an acetic buffer (pH 4.6) and 3 ml of a saturated aqueous Tropaeolin 00. The resulting mixture is then extracted with chloroform (4x5 ml). The combined extracts are acidified with 2 ml of an acid reagent (1 ml concentrated H_2SO_4 and 99 ml methanol) and diluted to 25 ml with chloroform. The alkaloid is then determined spectrophotometrically *at 545 nm* and calculated from a standard curve (89).

The above method was applied for the determination of small amounts of hyoscyamine and scopolamine in crude drugs (90):
These alkaloids were first separated by paper

chromatography with the solvent n-butanol-glacial acetic acid (10:1), saturated with water. After development, the spots were eluted, treated and determined as above.

Tropane alkaloids including scopolamine can be determined colorimetrically as follows (91):
Alkaloidal salt (1 mg) is dissolved in water (100 ml). To an aliquot of this (1 ml), mM bromocresol purple (3 ml) and buffer solution of pH 4.0 (2 ml) are added. The so produced color is extracted with chloroform (4x5 ml). The combined extracts are diluted to 10 ml with chloroform.
The absorbance of this at the absorption maxima *(405 to 410 nm)* is measured. The alkaloid content is then calculated from a calibration graph. Hydrolysis products of alkaloids do not interfere unless present in threefold amounts.
The relative error of the method does not exceed ± 1%.

The hydrobromide and methylbromide salts of scopolamine in tablets can be assayed by the following method (92).
Powdered tablets are extracted with water and suitably diluted. To an aliquot (5 ml ≡ ≅3 mg of alkaloid) in 50 ml flask placed in an ice water bath, saturated aqueous hydroxylammonium chloride solution (1 ml) and 10.5 M KOH (1 ml) are added, mixed and set aside for 1 hour. 4 M HCl (2 ml) is added to the mixture to give a pH range of 1.2 to 1.4. 0.37 M $FeCl_3$ solution in 0.1M HCl (1 ml) is then added and mixed. The flask is removed from the bath, gas evolution is allowed to subside and the extinction of the product is then measured at *540 nm* against a blank.

Other colorimetric methods have also been reported (93-99).

9.5.2 Ultraviolet Determinations

A physical method is described for the determination of scopolamine HBr in diphenhydramine-scopolamine tablets (100).
20 crushed tablets are shaked with 10 ml absolute ethanol for 5 minutes and centrifuged.

The supernatant solution (8 ml) is evaporated on a steam water bath until dryness, the residue is dried overnight in a vacuum. The dried residue is warmed at 50° for 15 to 20 minutes 1 ml of NaCl-saturated diethylacetamide and centrifuged. The resulting solution is examined spectroscopically in the region of 11 to 12 μ using a solvent blank.
An accuracy > 90% is atained and the method is unaffected by a high proportion of diphenhydramine HCl.

Systematic toxicological analysis by spectrophotometric method has been published (101).
The sample of tissue is homogenized with 0.1N HCl (25 ml). The homogenate is extracted on a water bath with 95% ethanol (75 ml) and 10% Na_2WO_4 (2 ml). The residue is dissolved in MaIlvain's buffer at pH 7.0 and extracted with chloroform (50 ml). The separated chloroform layer is then extracted with 0.1N HCl (100 ml). The characteristic UV absorption curves for 30 alkaloids including scopolamine in dilute HCl are presented.

Scopolamine hydrobromide as well as atropine sulfate in eye-drops, each can be determined by UV technique (102). Both alkaloids show maxima at *186 nm*. To determine scopolamine, dilute 1 ml sample to 1 lt. with water, 20 ml of this solution is further diluted to 100 ml with water. The extinction at *186 nm* is measured against water. Beer's law is obeyed over the range of 0 to 6 μg of scopolamine per ml. The results obtained are within 1% of those obtained by extraction methods.

Several alkaloids including scopolamine can be assayed in aqueous solutions of their salts particularly ampoules by UV spectrophotometric method (103). The extinction of the diluted sample is determined at the wavelength for maximum absorption *(257 to 286 nm)*.

Scopolamine in scopolamine butylbromide can be determined as follows (104).
A solution of the sample in water (2 g in 4 ml) is treated with aqueous ammonia (1 ml), saturated with sodium chloride and extracted with a

mixture of chloroform-carbontetrachloride (1:2) (5 x 10 ml). The combined extracts are dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. The residue is extracted with carbon tetrachloride (5 x 10 ml) and this extract is again evaporated to dryness under reduced pressure. The residue is then dissolved in ethanol (10 ml). An aliquot of this (1.5 ml) is diluted with water (to 100 ml) and the extinction of the diluted solution is measured at *205 nm* against 1.5% ethanol in water. A standard solution is prepared by subjecting scopolamine (10 mg) to the same procedure.
The recovery of scopolamine is 96.5±1.63%, and the results agree with those obtained by GLC of the sample (after trimethylsilation) on a column of 1.5% of SE-30 on Chromosorb W at 220° using N as a carrier gas.

Unit-dose assay of tropine alkaloids can be performed by charge transfer complex technique (105).
Samples of powdered tablets, injection solutions, eye drops are extracted with 1,2-dichloroethane. A volume of the resulting extract (containing 0.05 to 0.15 mg of alkaloid) is mixed with mM-iodine in dichloroethane (1 ml). The mixture is then diluted with dichloroethane (10 ml). The extinction of the charge transfer complex is measured at the maximum of *280 nm* or at *295 nm* against a reagent blank. The coefficient of variation was 1.6 to 2.8%.

9.5.3 Infrared Determinations

The application of IR spectrophotometry to the quantitative determinations of atropine and scopolamine has been reported (106). The pressed KBr pellet technique was applied in these determinations. Recoveries from standard mixtures showed a mean value of 104% for atropine and 98.2% for scopolamine.

IR spectroscopy has been recommended for the identification of scopolamine (107). To obtain reproducible spectra and avoid polymorphic effects, the following technique was performed.

An ammonical aqueous solution of the tablets or injections of scopolamine was extracted with chloroform and this extract was applied to a column of Celite impregnated with NaBr and H_3PO_4. The hydrobromide so formed was eluted with water saturated chloroform. The eluate was evaporated, the residue was mixed with KBr and compressed into a disc for IR identification.

9.5.4 GLC-Mass Spectrometric Determination

A GLC-mass spectrometric method for sub-microgram (50 pg/ml) assay of scopolamine in plasma and urine has been developed (108). The method used a deuterated internal standard to minimize variability in absolute recovery in the extraction procedure.
Scopoline and deuterated scopoline were formed from the base-catalyzed hydrolysis of scopolamine and the internal standard and were analyzed as the heptafluorobutyrates, using a GLC-mass spectrometric system by monitoring the *m/e 138 and 141* fragments respectively.

Procedure:
A plasma or urine sample (2-4 ml) is mixed with (-)-N-trideuteroscopolamine as internal standard and the mixture is adjusted to pH 9.75 with 1M carbonate buffer (2 ml) and extracted with methylene chloride.
The extract is purified by solvent partition, then the alkaloids are hydrolyzed to scopoline and deuteroscopoline by heating with 5N NaOH at 50° for 30 minutes. The substances are further purified by solvent partition and converted to their heptafluorobutyryl derivatives. The derivatives are then submitted into the following GLC-mass system:
Silanized glass column (1.8m x 2mm i.d.) packed with 3% OV-17 (for plasma samples) or with 1% OV-225 (for urine samples) on Gas-Chrom Q (100-120 mesh), operated at 95°.
The signal ratios at m/e 138 and 141 are measured. The procedure is sensitive to 50 pg ml^{-1}.
The coefficient of variation at the level of 250 pgm^{-1} (5 determinations on different days) was 2.5% (108).

9.6 Chromatographic Methods

9.6.1 Paper Chromatography

Clarke (17) described the following three systems for the identification of scopolamine and derivatives:-

I) Whatman No. 1, sheet 14 x 6 inches, buffered by dipping in a 5% solution of sodium dihydrogen citrate, blotting and drying at 25° for one hour. It can be stored indefinitely.
A solvent composed of 4.8 g of citric acid in a mixture of 130 ml of water and 870 ml of n-butanol was used (109).
The following Rf values were reported:

Scopolamine	0.23
Scopolamine butylbromide	0.93
Scopolamine methonitrate	0.20

Location reagent: Iodoplatinate spray.

II) Whatman No. 1 or No.3, sheet 17 x 19 cm, impregnated by dipping in a 10% solution of tributyrin in acetone and drying in air.
Solvent system: acetate buffer (pH 4.58).
Equilibration: The solvent in a beaker was equilibrated in a thermostatically controlled oven at 95° for about 15 minutes (110).
Rf value of scopolamine = 0.93.
Location reagent: as for I.

III) Chromatogram: as for II.
Solvent system: phosphate buffer (pH 7.40).
Equilibration: The solvent in a beaker was equilibrated in a thermostatically controlled oven at 86° for about 15 minutes (111).
Location reagent: as for I.
Rf values: for scopolamine 0.57; for scopolamine methonitrate 0.93.

Paper chromatography has been used for quantitative determinations of the tropane alkaloids or for their separation from crude drugs prior to determination.

The following system was reported for the microdetermination of solanaceous alkaloids (112) Ethereal extract of a crude drug was applied to strips of filter paper impregnated with potassium chloride solution. The chromatograms were developed in the solvent n-butanol-aqueous

HCl by the ascending technique for 20 hours. After development, the chromatograms were dried, sprayed with modified Dragendorff's reagent and areas of the spots were measured. The relative concentrations of the individual alkaloids were calculated by reference to calibration curves. Rf values : hyoscyamine 0.85; scopolamine 0.63 and atropine 0.34 (112).

(-)-Scopolamine can be determined in the presence of excess morphine and ethylmorphine (113). After separation of the mixture on paper chromatograms, the spots were measured by means of a densitometer and the constructed curves evaluated planimetrically.

Tropane alkaloids in crude drugs can be qualitatively identified, separated by paper chromatography and quantitatively assayed by colorimetric determination (114). Whatman no.3 filter paper was used and the chromatograms were developed with the solvent water saturated n-butanol-glacial acetic acid (25:1) for 18 hours. Corresponding areas on the chromatograms were identified, cut off, eluted with ethanol and determined colorimetrically.

Other paper chromatographic systems have been reported (115-118).

9.6.2 Paper Electrophoresis

Measurements of the electrophoretic mobility of 68 different alkaloids including scopolamine were reported (119). This was performed in an LKB apparatus on Whatman no. 1 paper (18 x 46 cm) at 8v/cm for 3 hours in the presence of universal buffers at various pH values. The relative displacements (rd) for scopolamine at various pH values were reported as follows:

pH 2.3, 4.3, 6.4, 8.2, 10.5 and 11.4
rd 67, 66.5, 104, 60, 33 and 13

The alkaloids were identified in filtered UV or by spraying with various alkaloidal reagents.

9.6.3 Thin Layer Chromatography (TLC)

Many TLC systems have been reported for the identification of scopolamine and other tropane alkaloids. Several of these systems are presented in table 7.

Table 7 : TLC of Scopolamine

	Chromatogram	Solvent System	Rf	Ref.
1.	Silica gel G (0.25 mm layers)	Methanol-strong ammonia (100:1.5)	0.54	(17,120)
2.	Silica gel G 250 μm thick, dipped in 0.1M KOH in methanol	Chloroform-methanol (90:10)	0.39	(16)
3.	Silica gel G layers	Chloroform-acetone-diethyl-amine (5:4:1)	0.56	(121)
4.	" "	Chloroform-diethylamine (9:1)	0.6	(121)
5.	" "	Cyclohexane-chloroform-diethylamine (5:4:1)	0.19	(121)
6.	" "	Benzene-ethylacetate-diethylamine (7:2:1)	0.34	(121)
7.	Aluminium oxide G. layers	Chloroform	0.30	(121)
8.	Basic silica gel G.	Methanol	0.52	(121)
9.	Silica gel G layers	Ethylacetate-ethanol-dimethylformamide-diethylamine (12:6:1 :1)	0.73	(122)
10.	Alkaline silica gel G layer dipped in 0.5N KOH	70% Ethanol-25% ammonia (99:1)	0.83	(122)

TLC has also been used for quantitative determinations of the tropane alkaloids.

Belladonna alkaloids can be determined by quantitative TLC (123).
Samples of *Belladonna* are extracted according to the method of Pharm. Helv. V (Swiss Pharmacopeia V). The alkaloids so extracted are then separated on kieselgel G plates with the solvent acetone-aqueous ammonia (19:1). After development, the chromatograms are dried, sprayed with Dragendorff's reagent and the areas of the spots are measured.

The results obtained by this procedure agree with those obtained by the modified Pharm. Helv. V methods (123).

Hyoscyamine and scopolamine can be determined by direct photodensitometry on thin layer chromatograms (124).
After extraction of alkaloids from powdered hyoscyamus (4 g) and purification, an aliquot is applied to 0.5 mm layers of silica gel G which developed for ≅ 1 hour with the solvent ahydrous methanol-aqueous ammonia (200:1). After development, the plates are dried at room temperature, sprayed with modified Dragendorff's reagent. The spots are scanned at *490 nm* with a recording and integrating densitometer.
The integrator count is a rectilinear function of the weight of alkaloid over the range of 4 to 65 μg for hyoscyamine and 7 to 60 μg for scopolamine. The results agreed well with those obtained by the spectrophotometric method of Freeman (81).

Tropane alkaloids in galenical preparations *(syrups, tablets, suppositories)* can be determined colorimetrically after separation on TLC plates (125).
The alkaloids are extracted with ammonical ethyl-ether-chloroform mixture. The organic layer of the extract is evaporated and dissolved in ethanol. This is applied into thin layers of silica gel GF_{254} which developed in the solvent ethanol-water-triethanolamine (5:4:1). After development, the zones are scrapped from the plates and dissolved in dilute nitric acid. The acid solution is evaporated and the resulting residue is dissolved in anhydrous ethanol-acetone (3:97), 3% methanolic KOH is added and the extinction of scopolamine at *575 nm* is measured within 1 minute. The concentration of the substance is determined by reference to a calibration graph.

Quantitative determination of scopolamine by TLC-fluorimetry has been described (126).
The method depends on measurement of the fluorescence of scopolamine spots after heating with H_2SO_4.
The fluorescence is measured with a Turner fluorometer fitted with a recorder. TLC system is as follows:

Kieselgel G layers, and the solvent consists of anhydrous ethanol-concentrated H_2SO_4 (39:7 by weight), length of run 7 cm. After development, plates are dried first in air and then by heating at 170° for 45 minutes.
(The absorbent must be free from substances that fluoresce under condition of the method). Rectilinear response was obtained up to a level of 6 μg alkaloid (126).

Stability of scopolamine HBr in eye drops can be indicated by TLC systems (127) : Scopolamine and its decomposition products scopine, aposcopolamine (apohyoscine) and tropic acid can be identified by TLC on kieselgel G, Rf values in five solvent systems are reported.

Other TLC system have also been reported (128,129).

9.6.4 Gas Liquid Chromatography (GLC)

Many GLC systems have been reported for the identification and quantitation of tropane alkaloids including scopolamine.

System I : This system was recommended to determine atropine and scopolamine quantitatively in their crude drugs (130).

Extraction : Alkaloids are extracted from a sample of powdered belladonna or stramonium (10g) by the method of USP XVII for total alkaloids of belladonna leaf.
2 μl aliquots of the extract are applied in duplicate.

Column condition : Glass (6 ft. x 0.075 inch i.d.) packed with 2.5% SE-30/S on acid washed silanized Chromosorb G (80-100 mesh). Temperature programmed from 150° to 275° at 6° per minute. Inlet port maintained at 315°.

Carrier gas : Helium (100 ml/minute).

Detection : Flame ionization.

Calculation : After development, the peak areas are measured.

The precision for each alkaloid is ≅ ±2.5% and the method is quantitative for amounts in the range 2 μg to 53 mg (130).

System II : The following system was reported for quantitative determination of scopolamine and atropine plus hyoscyamine in unit doses of tablets and elixirs (131).

Extraction : A unit dose form containing 6 μg scopolamine 100 μg atropine plus hyoscyamine is extracted. The extract and washing is evaporated to ≅ 0.1 ml and 1 to 2 μl are submitted to the system.

Column condition : Glass column (60cm x 3mm i.d.) containing 3% OV-17 on Gas Chrom Q (80-100 mesh), Column is maintained at 210°.

Carrier gas : Helium (60 ml/minute).

Detection : Flame ionization.

Calculation : The peak height ratio is measured and the concentration of each alkaloid is calculated from calibration graph.

The co-efficient of variation (10-determinations) are : for scopolamine ± 4.8%, for atropine plus hyoscyamine +2.5%.

System III : This system has been described as a simplified quantitative analysis of atropine and other alkaloids in scopolia extract including scopolamine. The method is also applied to gastrointestinal drugs of these alkaloids (132). The alkaloids (atropine, scopolamine and others) are separated as their trimethylsilyl derivatives. Diphenhydramine is used as internal standard.

Column condition : A column (1 m x 3 mm) packed with 0.75% of Dexsil 300 GC on Gas-chrom Q, column temperature is maintained at 180°.

Carrier gas : Nitrogen (40 ml min^{-1}).

Detection : Flame ionization.

Calculation : The calibration graphs (peak-height ratio vs alkaloid content) is rectilinear for 25 to 75 ng of atropine and 2.5 to 7.5 ng of scopolamine.

Many other GLC have been reported for the identification of scopolamine and other tropane alkaloids. Several of these systems are shown in table 8.

Table 8 : The GLC of Scopolamine

	Column condition	Carrier gas, Gas flow	Detector	Retention time (t_R) " Index (RI)	Ref.
I.	1% SE-30 on 100-120 mesh Anakrom ABS, 6 ft. x 4 mm i.d., borosilicate glass. Temperature 250°	Argon, 80 ml/minute	Argon ionization or Flame ionization	t_R 0.89 (relative to codeine)	(133)
II.	2.5% SE-30 on 80-100 mesh Chromosorb W, 5 ft. x 4 mm i.d., glass. Temperature 225°	Nitrogen, 50 ml/minute	Flame ionization H_2, 50 ml/minute, air flow 300 ml per minute	t_R 0.84 (relative to codeine)	(134)
III.	3% QF-1 on 100-120 mesh Anakrom ABS, Temperature 200°	Argon, 80 ml/minute	Flame ionization	t_R 10.7 (relative to diphenhydramine)	(133)
IV.	3% XE-60 silicone nitrile polymer on 100-120 mesh Chromosorb W. Temperature 225°	Nitrogen, 50 ml/minute	As for system II	t_R 1.39 (relative to codeine)	(17)
V.	5% SE-30 on 60-80 mesh Chromosorb W AW, 5 ft. x 1/8 inch i.d., stainless steel. Temperature 230°	Nitrogen, 30.7ml/minute	Flame ionization, Hydrogen 22 ml/ minute	t_R 0.84 (relative to codeine)	(135)

	Column condition	Carrier gas, Gas flow	Detector	Retention time (tR) " Index (RI)	Ref.
VI.	2.5% SE-30 on 80-100 mesh Chromosorb G (acid washed and dimethyl-dichlorosilane treated), 2m x 4mm i.d., glass. Temperature 100-300°.	Nitrogen, 45 ml/minute	-	RI 2303 (Reference = n-alkanes with an even number of carbon atoms)	(136)
VII.	3% Poly A103 on 80-100 mesh Chromosorb W HP, 1m x 4mm i.d., glass. Temperature 200°.	Nitrogen, 60 ml/minute	-	RI 2885 (Reference as in system VI).	(137)
VIII.	3% OV-17 on 100-120 mesh Chromosorb W-HP, 6 ft. x 2mm i.d., glass. Temperature 150-250°.	Nitrogen, 50 ml/minute; air, 120 ml/minute; hydrogen 2 ml/minute	N-FID	RI 2729 (Reference = methagualon)	(138)
IX.	3% OV-1 on 100-120 mesh Chromosorb W-HP, 6 ft. x 2mm i.d., glass. Temperature 150-250°, initial time 8 minutes.	as for system VIII	N-FID	RI 2329 (Reference = 2-amino-5-chloro-benzophenone)	(138)

Other GLC techniques have also been reported (127, 139-145).

9.6.5 High Performance Liquid Chromatography (HPLC)

System I : The following HPLC system has been applied to the separation and quantitative determination of tropane alkaloids including scopolamine (146).

Column : A stainless steel column (1m x 4.6mm i.d.) dry packed with Sil-X adsorbent.

Procedure : Samples were dissolved in methanol and injected directly into the column.

Mobile phase : A mixture of ammonium hydroxide (28% NH_3 by weight in water) and tetrahydrofuran in the ratio 1:100 v/v.

Detection : Differential Refractive Index detector having a range of 1.30-1.45 RI units and a high sensitivity UV detector (at 254 *nm*).

The average deviation in integrator counts, over a concentration range of 5-50μg was within ± 1% for all the alkaloids examined.

System II : This system is a reversed-phase liquid chromatography of basic drugs (including scopolamine) and pesticides with a fluorigenic ion pair extraction detector (147).

Column : Two types were used:

- 10 cm x 3 mm (i.d.) packed chemically bonded 10μm silica gel of the diol and CN type of Lichrosorb RP-2, RP-8 and RP-18.
- Micro-Pack CN-10 column (30 cm x 4 mm i.d.).

Solvent : 25% methanol in water containing 0.1N NaH_2PO_4 (pH 3.5).

Detection : Ion pair extraction detector for the sensitive and selective fluorescence detection.

System III : The following HPLC system was recommended for analysis and quantitation of belladonna alkaloids and can be applied to elixirs, tablets and capsules of these alkaloids containing phenobarbitone (148).

Extraction : The sample is treated with H_2SO_4 and mixed with CH_2Cl_2, after shaking, the organic layer is discarded. Carbonate buffer solution (pH 9.4) is added to the aqueous phase and the mixture is extracted with CH_2Cl_2. The organic phase is filtered, evaporated, theophylline (as internal standard) is added and the solvent evaporated to dryness. The residue is treated with MeOH-HCl and evaporated to dryness, the residue is re-dissolved in methanol and the methanolic solution is again evaporated to dryness. The final residue is dissolved in water and applied to the following HPLC system.

Column : A stainless steel (25cm x 4mm) of Spherisorb ODS (5μm).

Mobile phase : Water-methanol (21:10, pH 2.0) containing tetramethylammonium phosphate (0.8 ml $min.^{-1}$).

Detection : UV at 220 nm.

- The calibration graphs were rectilinear in the range of 0-0.5 mg ml^{-1} (hyoscyamine plus atropine), 0-0.32 mg ml^{-1} (scopolamine).
- The coefficient of variation was 1.4% for scopolamine and 0.5% for hyoscyamine.
- The detection limit was 0.02μg for each alkaloid.
- Recovery of the compounds was 93.0 to 106.1% (148).

Many other HPLC systems have been reported for identification and quantitation of scopolamine and other tropane alkaloids. Several of these are presented in the following table 9.

Table 9 : HPLC of Scopolamine

Column	Mobile Phase	Internal Standard	Detector	Ref.
1- Stainless steel, (30cm x 4mm), packed with μ-Bondapak C_{18}	0.1M Na decyl sulfate-0.1M NH_4NO_3 in aqueous 60% methanol or 55% ethanol.	Benzphetamine hydrochloride	UV at *254nm*	(149)
2- A 12.5cm x 4.9mm packed with silica (spherisorb S_5 W, 5μm) Stainless-steel	A solution containing 1.175 g (0.01M) of ammonium perchlorate in 100 ml methanol adjusted to pH 6.7 by 1 ml of 0.1M NaOH in methanol	————	UV/electro-chemical K' 1.1	(150)
3- A 250x4 mm² of RP-18 (10μm)	156 g acetonitrile + 344 g phosphate buffer (4.8 g 85% H_3PO_4 and 6.66 g KH_2PO_4) pH 2.3 flow rate 1 ml/minute (isocratically).	MPH = 5-(p-methyl-phenyl)-5-phenyl-hydrantoin	UV at *220nm* Rel. t_R of scopolamine N-butylbro-mide 1.02	(138)
4- A 15cm x 4.6mm of Shodex ODS pak.	50% methanol (adjusted to pH 4.0 with acetic acid) containing 5mM sod. heptane-1-sulfonate	————	UV *215nm* (limits 0.15μg)	(151)
5- A 30cm x 3.9mm of μ Bondapak C_{18} (10 μm)	3% aqueous acetic acid - methanol (7:3 for detection, 3:1 for determination)(1ml min^{-1})	L-Hyoscyamine, scopolamine HBr.	UV *254nm*	(152)

Other HPLC have also been reported (153-155).

Further important references on various chromatographic techniques were found in the literatures (156-158).

9.7 Radioligand Assay Methods

A simple and sensitive radioligand binding assay is described for the determination of scopolamine in human urine. As a measure for the drug concentration, the quantitative displacement of scopolamine of tritiated quinuclidinyl benzylate from rat brain receptors was used. The assay is sensitive to concentrations as low as *1.2 ng/mL*. It can be performed easily and quickly, also no prior extraction procedure is required. Scopoline and scopine, possible metabolites of scopolamine do not interfere with the assay (54).

Anticholinergic drugs including scopolamine indicating that their incubation with muscarinic receptor at 0° before and after addition of the radiolabelled ligand [^{3}H] dexetimide can provide lower detection limits (by a factor of 2.5 to 9) with coefficient of variation 3 to 9% (159).

Acknowledgement

The authors would like to thank Mr. Uday C. Sharma, Department of Pharmacognosy, College of Pharmacy, Riyadh, Saudi Arabia for his valuable and sincere efforts in typing this manuscript.

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SULFOXONE SODIUM

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1. Introduction

Sulfoxone sodium belongs to the class of sulfones which are derivatives of 4,4'-diaminodiphenylsulfone (dapsone, DDS). The sulfones were synthesized on analogy of sulfonamides. Dapsone was found in 1937 to be thirty times more active as sulfanilamide when used in streptococcal infections in mice. In the 1940s sulfones were found to be effective in suppressing experimental infections with the tubercle bacillus and for rat leprosy. The usefulness of sulfones in the chemotherapy of human tuberculosis was very limited, but their clinical trials in human leprosy were successful.[1,2] The sulfones are presently the most important drugs for the treatment of leprosy. Sulfoxone sodium acts through its conversion to dapsone in the body.

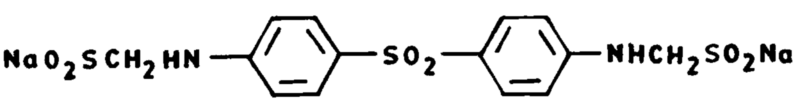
2. Description

2.1 Name, Formula and Molecular Weight

Sulfoxone sodium is also known as sodium sulfoxone, aldesulfone sodium, and as sulfoxydiasulfone sodium. Chemically, it is disodium [sulfonylbis(p-phenyleneimino)]dimethanesulfinate. Other chemical names are: disodium salt of [sulfonylbis(1,4-phenyleneimino)]bismethanesulfinic acid; disodium 4,4'-diaminodiphenylsulfone bisformaldehydesulfoxylate; disodium formaldehydesulfoxylate diaminodiphenylsulfone.

The CAS registry number for sulfoxone is 144-76-3, and for sulfoxone sodium is 144-75-2.

The proprietary name for sulfoxone sodium is Diasone or Diasone Sodium.



$C_{14}H_{14}N_2Na_2O_6S_3$ Molecular Weight: 448.43

2.2 Appearance, Color and Odor

A white to pale yellow powder with a characteristic odor. Sulfoxone sodium USP is a mixture of disodium [sulfonylbis(p-phenyleneimino)]dimethanesulfinate and suitable buffers and inert ingredients. It contains not less than 73.0 per cent and not more than 81.0 per cent of $C_{14}H_{14}N_2Na_2O_6S_3$, calculated on the dried basis.

3. Physical Properties

3.1 Optical Rotation

Sulfoxone sodium exhibits no optical activity.

3.2 Melting Range

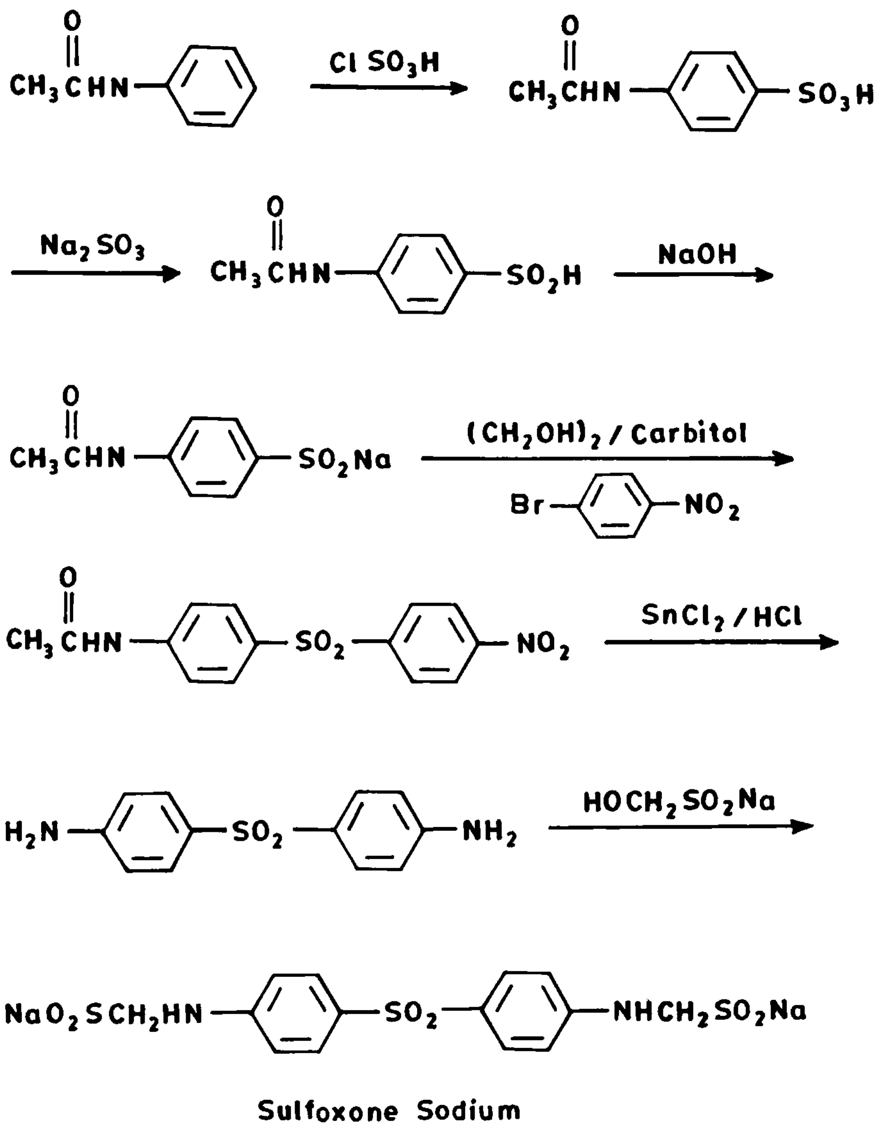
The melting range[3] of sulfoxone sodium after drying at 100-110° is 263-265° (decomp). Another report mentions the melting point of amorphous product as 268° (decomp).[4]

3.3 Solubility

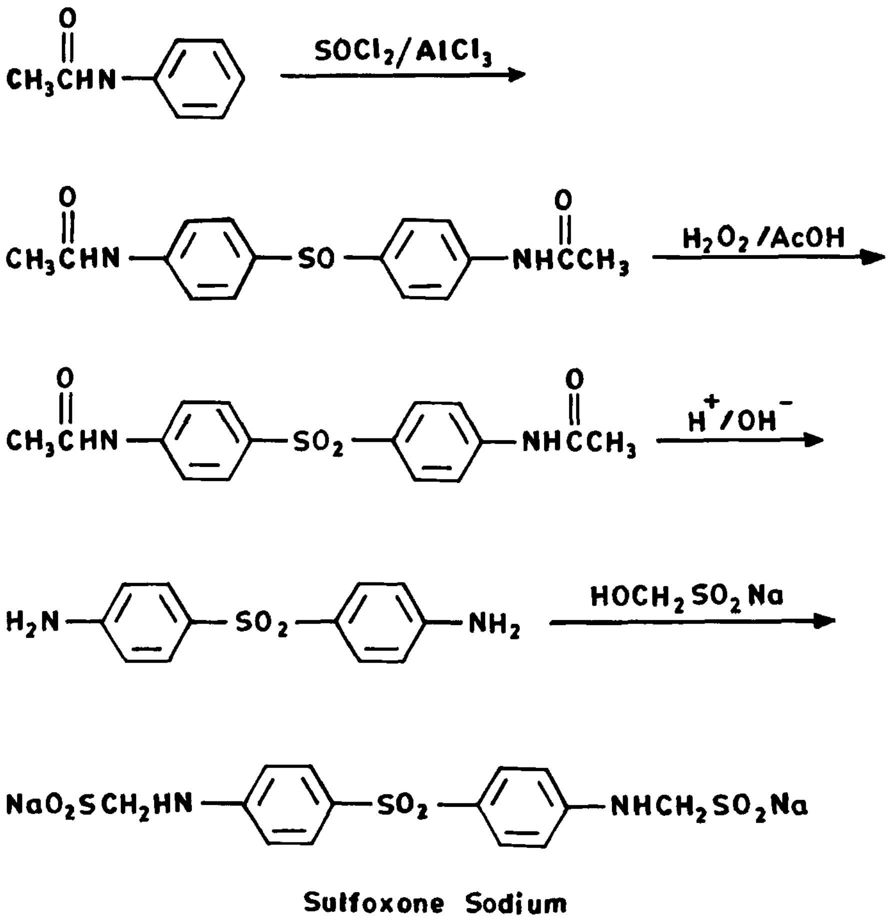
Sulfoxone sodium is soluble (1 in about 14) of water yielding a clear to hazy pale yellow solution.[5] A 10% solution in water has a pH of 10.5 to 11.5. Sulfoxone sodium is very slightly soluble in alcohol, chloroform, and ether.[5]

4. Synthesis

Sulfoxone sodium was prepared by Raiziss *et al.*[3] by adding finely powdered sodium formaldehyde-sulfoxylate ($OHCH_2SO_2Na$) to a solution of 4,4'-diaminodiphenylsulfone in specially denatured alcohol, and refluxing the mixture for five hours on a steam bath. The sulfoxone sodium is obtained as a tetrahydrate amorphous powder in 95% yield by this method. Sulfoxone sodium was also independently prepared using a different method by Bauer and Rosenthal.[6-8] In the latter method the combination of 4,4'-diaminodiphenylsulfone



Scheme I – Synthesis of Sulfoxone Sodium



Scheme II - Alternate Synthetic Route to Sulfoxone Sodium

with sodium formaldehydesulfoxylate was done in glacial acetic acid. Upon addition of alcohol to the neutralized aqueous solution the reaction product crystallized as needles with two moles of water of crystallisation.[8] Schemes (Scheme-I,[9] Scheme-II[4]) for complete synthesis of sulfoxone

sodium have also been described.[4,9,10] Other synthetic routes to 4,4'-diaminodiphenylsulfone (dapsone) have been reported earlier.[11] Preparation of a salt of sulfoxone sodium with antimalarial amodiaquin, useful against <u>Plasmodium berghei</u> infection, has been reported.[12]

5. Stability

The combination of 4,4'-diaminodiphenylsulfone and sodium formaldehydesulfoxylate in sulfoxone sodium is reported[3] to be firm and can withstand the splitting effect of various agents. In sealed glass ampoules under high vacuum sulfoxone sodium remains water-soluble indefinitely. Exposed to air for several days it undergoes some change that renders part of it insoluble, but mixed with small amount of sodium bicarbonate it remains unchanged for several months and is easily soluble in water.[3,8] A 0.4% hydrochloric acid solution at 37° does not, over a period of 2 hours, cause splitting off of the formaldehydesulfoxylate or liberation of 4,4'-diaminodiphenylsulfone. Acetic acid or carbon dioxide merely causes precipitation from alkaline solutions and the precipitate redissolves on adding alkali.[3] United States Pharmacopeia recommends that sulfoxone sodium be preserved in tight, light-resistant containers under the atmosphere of nitrogen, in a freezer.[13]

6. Biological Activity

Sulfoxone sodium was prepared in a successful attempt to reduce the toxicity of 4,4'-diaminodiphenylsulfone (dapsone), and was reported to have a therapeutic index considerably greater than that of the latter.[14] Sulfoxone sodium was found to be decidedly less toxic than dapsone.[15,16] Sulfoxone sodium was also found to be an effective agent in experimental tuberculosis.[17-26] It was, however, found ineffective clinically as suppressive in human tuberculosis.[27] There are several reports on the effectiveness of sulfoxone sodium in experimental toxoplasmosis.[28-31] Sulfoxone has also been studied for experimental meningococcus infection in mice.[32] It showed no

bacteriostatic activity *in vitro* against *Paracoccidioides brasiliensis*[33], *Eberthella typhosa*[34], and on acid-fast bacilli isolated from lepers or on Stefansky's bacillus.[35] Powell *et al.*[36] have indicated a potential role of sulfoxone in the prevention and treatment of infections with chloroquin-resistant *Plasmodium falciparum*. There are other reports on different biological activities involving sulfoxone sodium.[37-40] Pharmacology and other related aspects of sulfoxone sodium have been reviewed.[41,42]

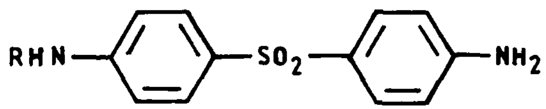
Therapeutically sulfoxone sodium is used for the treatment of leprosy. It has actions similar to those of dapsone and is given to patients who experience severe gastrointestinal side-effects with dapsone.[5] In higher doses it is used for dermatitis herpetiformis. The mechanism of action of sulfones is probably similar to that of the sulfonamides since both interefere with incorporation of *p*-aminobenzoic acid into dehydrofolate.[1,2] The possible relation between the fact that sulfoxone enters washed mycobacterial cells and the mechanism of bacteriostasis has been discussed.[43] Total protein, total globulin, pseudoglobulin and euglobulin concentrations of the serums of lepers varied greatly with the progress of the disease. Higher values for these fractions have been found in advanced cases than in earlier cases. It has been observed that after six months treatment with sulfoxone most of the protein fractions decreased, especially euglobulin.[44] It has also been reported that compounds which inhibited phenoloxidase of *Mycobacterium leprae* also suppressed its multiplication in mice, suggesting that phenolase might be of metabolic significance to the organism.[45] Electron micrographs of AI-shadowed *M. leprae* from patients treated with sulfoxone showed swelling of the cytoplasm and a granular state of *M. leprae* and a disappearance of the peripheral halo.[46] Swislocki *et al.*[47] have discussed the mechanism of effects of sulfones, and have reported that sulfoxone increased the activities of adenylate cyclase and protein kinase in erythrocytes.

Regarding the mechanism of action of sulfoxone sodium in the treatment of dermatitis herpetiformis,

it has been observed[48] that the drug inhibits both neutrophil iodination and cytotoxicity for tumor cells. This may represent an important mechanism by which sulfoxone sodium produces its therapeutic effect when used to treat inflammatory skin disease.

7. Metabolism and Pharmacokinetics

Metabolism of sulfoxone sodium has been studied by Francis and Spinks.[49] The drug gets hydrolysed in the gastrointestinal tract to 4,4'-diaminodiphenylsulfone (dapsone; DDS). It was shown that the therapeutic effects of sulfoxone sodium are closely related to the blood concentrations of 4,4'-diaminodiphenylsulfone.[49,50] 4,4'-Diaminodiphenylsulfone after absorption is metabolised to a number of products[11]; monoacetyldapsone (MADDS) being one of the major metabolites.



4,4'-Diaminodiphenylsulfone (DDS),R=H

Monoacetyldapsone (MADDS), R=$-COCH_3$

Several pharmacokinetic studies have been done on sulfoxone sodium.[41,51-56] The concentration of sulfoxone in the blood of fortyseven patients receiving orally 1 g of the drug daily for 1 to 4 years, varied from 0 to 3.6 mg %; the maximum concentration in urine was 100 mg %.[51] The sulfoxone concentration in the urine of twentyone patients receiving 0.66 g daily was 32 mg %.[51] Determination in blood, urine, tissue and faeces of thirtytwo biopsied lepers treated with sulfoxone for 4 months to 7 years led to the conclusion that sulfoxone is retained in the body up to 2, sometimes up to 4 weeks after cessation of treatment.[53] Some amount of the drug was found in the faeces. Excretion occurs through the kidney rapidly but may be disturbed by abnormal intestinal or impaired

renal function. A tendency to storage in the skin exists for sulfoxone. Liver, spleen, kidneys and nerves also serve as organs of concentrations.[53] In another study[54] free and combined sulfone were found in the blood of 47.3% of the lepers 24 hours after the end of treatment with sulfoxone sodium. Intravenous injection of sulfoxone sodium in five times the doses used for human therapy caused hemolysis in dogs. The hemolysis seemed to be proportional to the rate of hydrolysis and liberation of 4,4'-diaminodiphenylsulfone. The blood concentration of 4,4'-diaminodiphenylsulfone reached its maximum 30 min after injection.[55]

The disposition of the water-soluble sulfones in man was determined earlier by applying the Bratton and Marshall procedure[57] for measuring aromatic amines.[58] Subsequently, analytical techniques were developed which could measure small amounts of 4,4'-diaminodiphenylsulfone in plasma.[59-62] This made possible a realistic assessment of the DDS levels obtained in patients treated with water-soluble sulfones. Peters _et al._[56] have studied the disposition of sulfoxone sodium in leprosy patients by determining the levels of dapsone (DDS) and monoacetyldapsone (MADDS) in plasma and urine by spectrophotofluorometric technique. Peak plasma levels of DDS were approximately 600 ng/ml five to eight hours after treatment with 330 mg sulfoxone sodium given orally. Because 330 mg sulfoxone contain 169 mg DDS, assuming complete hydrolysis to DDS, it could be estimated that an approximately three-fold higher molar dose of sulfoxone than of DDS was required to yield similar plasma levels of DDS. The urinary excretion pattern of DDS and MADDS after this drug was similar to that found after DDS treatment, but total DDS excretion was lower. Smith[41] also reported a large difference in the urinary excretion of total DDS during three days following oral sulfoxone and DDS by patients (55% versus 80%), and concluded that this was due primarily to relatively poor absorption of sulfoxone from the gastrointestinal tract. The results of investigation by Peters _et al._[56] indicate that regular sulfoxone therapy provides plasma levels of DDS that would be expected to be therapeutically effective and to protect patients from the development of DDS-resistant leprosy.

The bioequivalence requirements of sulfoxone sodium have been given.[63]

8. Toxicity

Bauer[8] studied the toxicity and therapeutic activity of sulfoxone sodium in streptococcal infection in mice, and has described 3 g per kg as minimum tolerated dose of the drug given subcutaneously; while minimum effective dose described is 0.2 g per kg. A marked reduction in the red blood cell count and hemoglobin level of the patients receiving 1 g of sulfoxone sodium for 120 days or more has been reported.[18] Primaquin sensitive erythrocytes were found to be unusually susceptible to hemolysis by sulfoxone sodium.[64,65] Several severe untowards effects caused by dapsone and its analogs have been described.[1,2] Besides frequent gastrointestinal and central nervous system disturbances, the most common untoward effect is hemolysis of varying degree. Development of peripheral neuropathy of thumbs and hand, without motor involvement, in a patient receiving sulfoxone sodium for dermatitis herpetiformis has been reported.[66]

The United States Pharmacopeia[13] gives the following safety test for sulfoxone sodium. Prepare a solution of sulfoxone sodium (1 in 10). Select 15 mice, each weighing between 20 and 25 g, and divide them into three groups of five each. Administer orally to each mouse of the first group 20 µL of the solution per g of body weight. In the same manner administer to each mouse of the second group and to each mouse of the third group 25 and 30 µL, respectively, per g of body weight: all of the mice of the first group, at least four of the second group, and at least two of the third group survive for 5 days.

9. Methods of Analysis

9.1 Elemental Composition

The elemental composition of sulfoxone sodium is as follows[67]:

Element	Per cent
C	37.49
H	3.15
N	6.25
Na	10.26
O	21.41
S	21.45

The composition of sulfoxone sodium dihydrate is reported[8] as follows:

Element	Per cent Calcd.	Per cent Found
N	5.78	5.64
S	19.86	19.80
Na	9.50	9.24

9.2 Identification Tests

9.21 Color Tests

The following color tests for the identification of sulfoxone sodium have been described:

A. A knifepoint of the material is placed on a square of blank newspaper and moistened with 2.4N hydrochloric acid and a drop or two of ethanol to aid solution if required, a deep yellow-orange color is produced when examined against a white background.[68] The test is based on lignin color reactions with amine compounds.

B. Feigl and Moscovici[69] have described a color test for the detection of sulfoxone sodium in very small quantities. The color reaction is based on the evolution of formaldehyde when the drug is warmed with concentrated sulfuric acid in a water bath. The evolved formaldehyde can

be detected in solution or in the vapor phase by means of chromotropic acid and concentrated sulfuric acid when a violet color is produced. The test has been recommended for the detection of sulfoxone sodium in pharmaceutical products.[70]

C. A bright red color is produced if sulfoxone sodium is heated with furfural-acetic acid and a drop of hydrochloric acid is added.[71]

D. An orange color is produced by treatment of sulfoxone sodium with dimethylaminobenzaldehyde.[71]

E. Sulfoxone sodium gives a blue color with a mixture of 1:1 of 10% aqueous ammonium molybdate and concentrated sulfuric acid.[71]

F. A pink color and some turbidity is produced with silicotungstic acid.[71]

G. A blue precipitate is produced with 0.25% methylene blue.[71]

H. Distillation of 0.5 g of sulfoxone sodium with 50 mL of 50% hydrochloric acid gives distillate which decolorises 0.1N iodine solution. The distillate gives a blue color upon addition of morpholine hydrochloride in concentrated sulfuric acid.[71]

I. In alcoholic solution sulfoxone sodium gives a violet color with gold chloride.[71]

J. Mixed with 5 drops of 10% ferric chloride and 5 drops of potassium ferricyanide, sulfoxone sodium gives a sky blue color.[71]

9.22 Pharmacopeial Tests

The United States Pharmacopeia gives the following identification tests for sulfoxone sodium[13]:

A. Transfer about 350 mg to a 100-mL volumetric flask, dissolve in water, dilute with water to volume, and mix. Transfer 10.0 mL of

this solution to a 100-mL volumetric flask, dilute with water to volume, and mix. Transfer 4.0 mL of the resulting solution to a suitable flask, add 0.5 mL of 3N hydrochloric acid, and hydrolyse in a boiling water bath for 30 minutes. Transfer the contents of the flask to a separator with the aid of water, add 2 mL of sodium hydroxide solution (1 in 10), and extract with three 25-mL portions of ethylene dichloride. Filter the extracts through a pledget of glass wool into a 100-mL volumetric flask and dilute with ethylene dichloride to volume. The ultraviolet absorption spectrum of the solution so obtained exhibits maxima and minima at the same wavelengths as that of a solution of USP Dapsone RS in ethylene dichloride having a known concentration of 5 μg per mL, concomitantly measured.

B. To 10 mL of a solution (1 in 100) add 1 mL of iodine TS and 2 mL of chloroform, shake vigorously, and allow the layers to separate: no color appears in either layer.

C. Ignite 200 mg, the residue responds to the tests for sodium.

9.3 Spectrophotometric Analysis

9.31 Colorimetric

A colorimetric method is employed to assay sulfoxone sodium.[13] It involves acid hydrolysis of sulfoxone sodium to liberate free amino groups followed by diazotization and coupling with N-1-naphthylethylenediamine to form an azo dye. The absorbance is measured spectrophotometrically at a wavelength of about 535 nm. A standard solution of dapsone is also used in the assay procedure which is as follows.

Transfer about 1 g, accurately weighed, of sulfoxone sodium to a 2000-mL volumetric flask, dissolve in water, dilute with water to volume, and mix. Pipet 4 mL of the solution into a 100-mL volumetric flask, add 1.0 mL of p-toluenesulfonic acid solution and 0.5 mL of 3N-hydrochloric acid, and heat the flask in a boiling water bath for 30 minutes. Cool, dilute with water to volume, and

mix. This solution is called as assay preparation.

Pipet 2 mL each of dapsone standard solution (prepared as directed[13]; each mL of this solution contains a known quantity of about 10 µg of dapsone, equivalent to 18.06 µg of $C_{14}H_{14}N_2Na_2O_6S_3$), the assay preparation, and water to provide the blank, into separate 25-mL volumetric flasks. To each flask add 2.0 ml of 3N-hydrochloric acid, 10.0 mL of water, and 2.0 mL of sodium nitrite solution, and mix. After 3 minutes, accurately timed, add 1.0 mL of ammonium sulfamate solution, and mix. Allow to stand for 2 minutes, accurately timed, dilute with N-1-naphthylethylenediamine dihydrochloride solution to volume, insert the stoppers in the flask, and mix. Allow to stand for 10 minutes, and concomitantly determine the absorbances of the solutions in 2-cm cells, at the wavelength of maximum absorbance about 535 nm, with a suitable spectrophotometer, using the blank to set the instrument. Calculate the quantity, in mg of $C_{14}H_{14}N_2Na_2O_6S_3$ in the sulfoxone sodium taken by the formula (448.43/248.30) (50C) (A_u/A_s), in which 448.43 and 248.30 are the molecular weights of $C_{14}H_{14}N_2Na_2O_6S_3$ and dapsone ($C_{12}H_{12}N_2O_2S$), respectively, C is the concentration, in µg per mL, of USP Dapsone RS in the dapsone standard solution, and A_u and A_s are the absorbances of the solutions from the assay preparation and dapsone standard solution, respectively.

9.32 Fluorometric

Fluorometric procedure has been employed in pharmacokinetic study of sulfoxone sodium in leprosy patients.[56]

9.4 Chromatographic Analysis

Orzech *et al.*[11] have described various chromatographic procedures for the analysis of dapsone, the active conversion product of sulfoxone sodium in the body.

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TENIPOSIDE

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1. HISTORY

Teniposide is a semi-synthetic derivative of epipodophyllotoxin and is closely related to etoposide [1]. It is used as a cytostatic in the treatment of several types of cancer. Teniposide was synthesized from podophyllotoxin in 1963, in the Sandoz Laboratories. Podophyllotoxin, the starting material for teniposide, is isolated from the dried roots and rhizomes of species of the genus *Podophyllum*, such as the may apple or American mandrake (*Podophyllum peltatum* L.) and *Podophyllum emodi* Wall.) [2].

The medicinal properties of podophyllin, the ethanolic extract of the roots and rhizomes of the above mentioned *Podophyllum* species have been known for more than 150 years. Podophyllin contains several podophyllotoxin derivatives, a number of which possess considerable anti-tumour activity. Podophyllotoxin itself proved to be the most active cytotoxic compound. However, the toxicity of naturally occurring podophyllotoxins prevents administration of doses high enough to give sufficient therapeutic effect. Therefore, a variety of derivatives were synthesized from natural podophyllotoxin in an attempt to find compounds with an acceptable therapeutic index [3-5]. Teniposide was one of the promising compounds.

2. DESCRIPTION

2.1. Nomenclature, Formula, and Molecular Weight

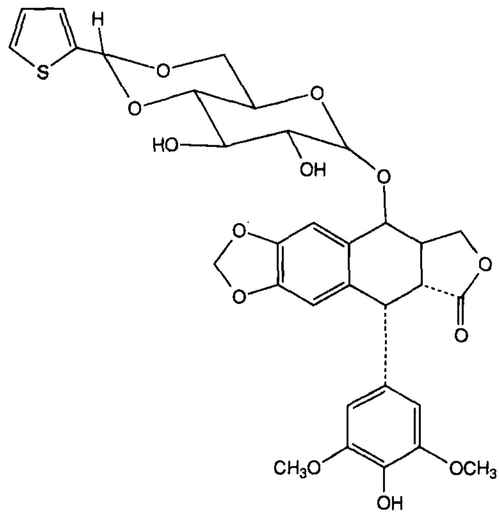
The generic name is teniposide (29767-20-2). Other names are VM 26, PTG, NSC 122819. The trade name of the drug is Vumon®.

The Chemical Abstracts' name is 4'-demethyl-1-O-[4,6-O-(2-thenylidene)-β-D-glucopyranosyl]epipodophyllotoxin (IUPAC) or 5,8,8a,9-tetrahydro-5-(4-hydroxy-3,5-dimethoxyphenyl)-9-[[4,6-O-(2-thienylmethylene)-β-D-glucopyranosyl]-oxy]-furo[3'4':6,7]naphtho[2,3-d]-1,3-dioxol-6(5aH)-one.

The molecular formula of teniposide is $C_{32}H_{32}O_{13}S$; its molecular weight is 656.7.

2.2. Appearance, Odour, and Colour

Teniposide is a white, odourless and amorphous powder.

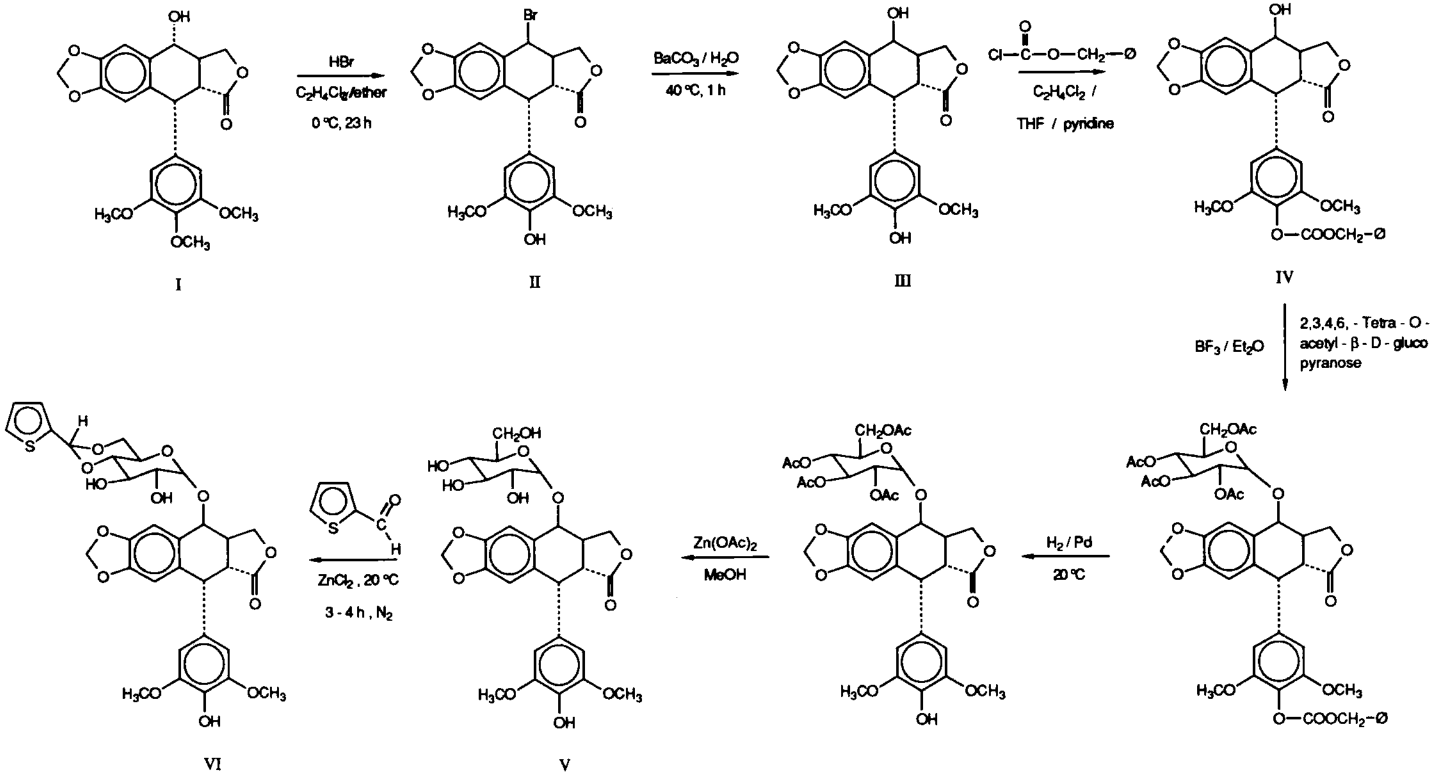


3. SYNTHESIS OF TENIPOSIDE

The synthesis of teniposide from naturally occurring podophyllotoxin I (Scheme 1) is described in ref. [3-5]. Podophyllotoxin is treated with HBr in 1,2-dichloroethane, resulting in 1-bromo-1-desoxyepipodophyllotoxin, which demethylates to 1-bromo-4'-demethylepipodophyllotoxin (II) when the reaction mixture is kept at 0°C for about 24 hours. By treatment of II with $BaCO_3$ in an acetone/water mixture, the bromine is replaced by a hydroxyl group, resulting in 4'-demethylepipodophyllotoxin (III). After protection of the phenolic hydroxyl with benzyl chloroformate, the 1-OH group is coupled with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranose. The sugar moiety probably enters from the less hindered side, because glycosidation of podophyllotoxin itself also results in an epi product [4].

The protecting group at the 4'-OH is removed by hydrogenolysis with H_2/Pd and the acyl groups by hydrolysis with $Zn(OAC)_2$ in methanol. During the hydrolysis, about 30% of the compound is converted into a mixture of the hydroxy acid (by opening of the lactone ring) and the *cis* lactone. These products are easily removed by crystallization.

The last step in the synthesis is the reaction with 2-thiophene carboxaldehyde, with $ZnCl_2$ as a catalyst. Of the O-4,6 cyclic acetals, the isomer with the equatorial thienyl group predominates.



Scheme 1. Synthesis of teniposide.

Minor quantities of the axial isomer are eliminated in the purification procedure [5].

4. PHYSICAL PROPERTIES

4.1. Ultraviolet Spectrum

The ultraviolet spectrum of a 71 μM solution of teniposide in absolute methanol (Figure 1) shows an absorption maximum at 283 nm. The specific extinction ($E^{1\%}_{1cm}$) at 283 nm is 64.1 (ε = 4209 $l.mol^{-1}.cm^{-1}$) [6]. The ultraviolet spectrum was recorded with a double beam Shimadzu Spectrophotometer UV-200 in a 1 cm silica cell.

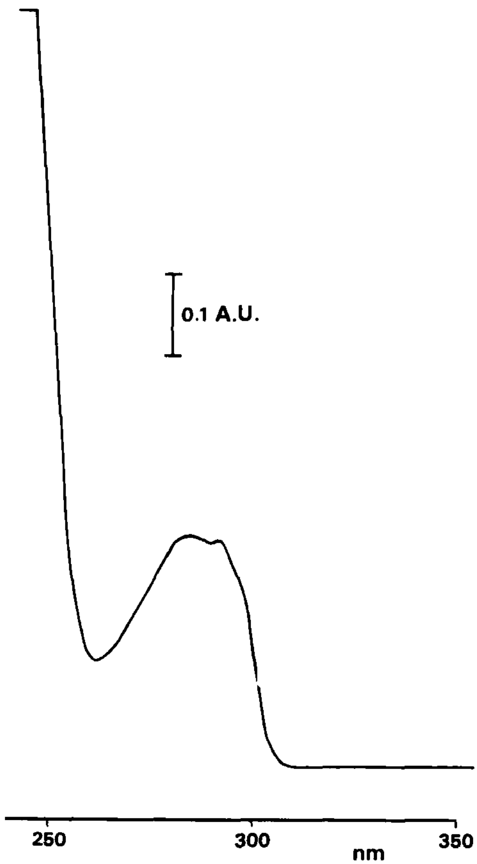


Figure 1. The ultraviolet spectrum of teniposide (71 μM) in methanol.

4.2. Infrared Spectrum

The IR spectrum of teniposide (KBr tablet) is shown in Figure 2. The spectrum was recorded with a Jouan-Jasco IRA-1 grating infrared spectrometer.

Characteristic bands are the carbonyl stretch vibration of the strained *trans* lactone ring at 1785 cm^{-1} the OH stretch vibration of the phenolic and sugar OH groups at 3540 and 3400 cm^{-1}, the aromatic bands at 1605, 1505 and 1485 cm^{-1} and C-O stretch vibrations at 1230 and 1100 cm^{-1}.

4.3. Fluorescence Emission Spectrum

The fluorescence emission spectrum of teniposide (Figure 3) was recorded with a Kontron SFM 25 fluorimeter. An excitation wavelength of 295 nm and a scan rate of 60 nm/min were used.

4.4. Nuclear Magnetic Resonance Spectrum

The proton NMR was recorded in deuterochloroform containing a drop of dimethyl sulfoxide-d_6, with a Bruker AM-500 spectrometer at a frequency of 500.14 MHz. The internal standard was DMSO (at 2.49 p.p.m.). The spectrum between 2.7 and 5.3 p.p.m. is reproduced in Figure 4.

Chemical shift assignments (Table I) were made on the basis of integrated intensity measurements and comparison with the spectrum of etoposide [7]. Coupling constants for ring C and D protons, and for the glucose and the thienyl moiety are given in Table II.

The natural abundance ^{13}C NMR spectrum was recorded with a Bruker SP-200 WB instrument at a frequency of 50.3 MHz, with deuterochloroform containing a drop of dimethyl sulfoxide-d_6 as the solvent. DMSO (at 39.5 p.p.m.) was used as the internal standard. The proton-noise decoupled spectrum is reproduced in Figure 5; the spectral assignments are presented in Table III.

Since some of the chemical shift values differ only slightly, the assignments for the corresponding signals may be interchanged.

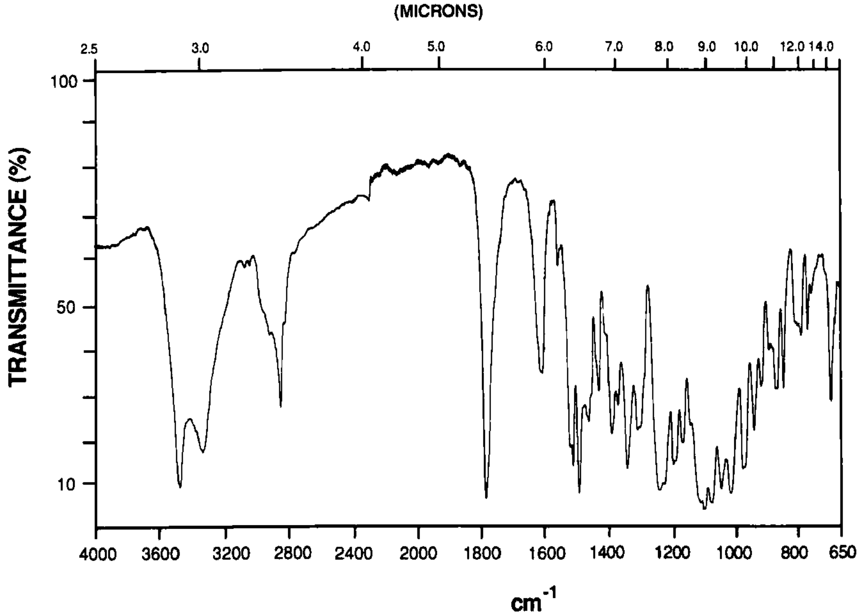


Figure 2. The infrared spectrum of teniposide.

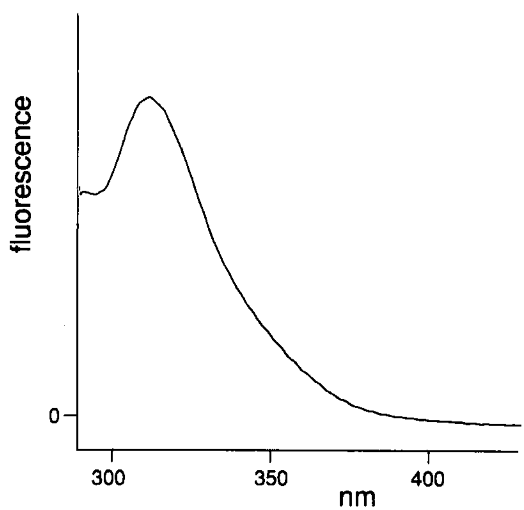


Figure 3. The fluorescence emission spectrum (not corrected) of teniposide (9.4 µM) in methanol.

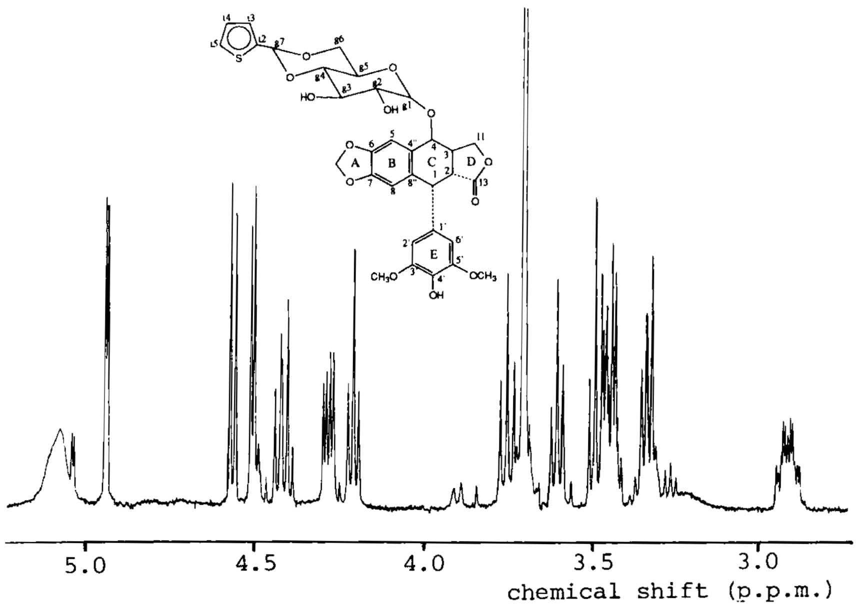


Figure 4. Proton NMR spectrum (2.7-5.3 p.p.m.) of teniposide.

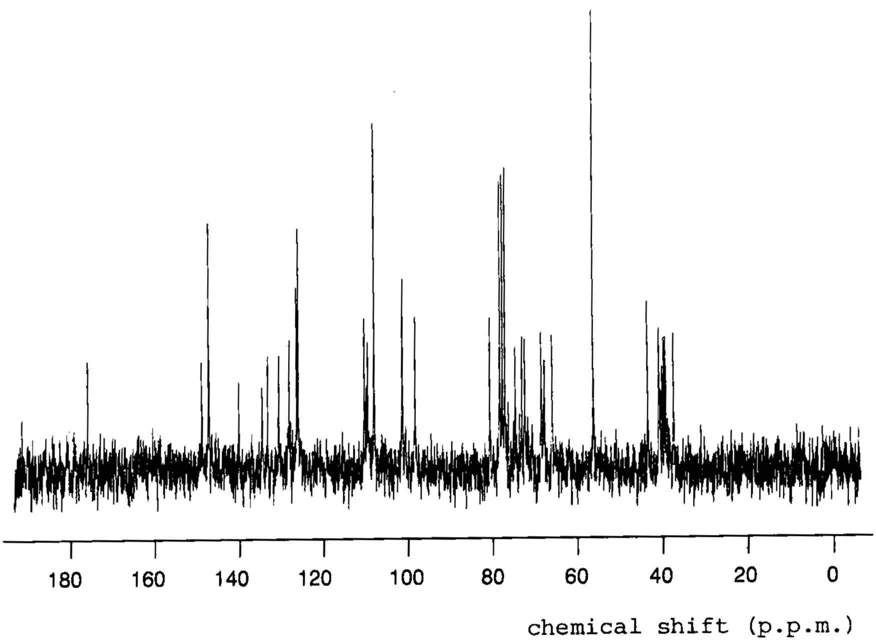


Figure 5. ^{13}C NMR spectrum of teniposide.

Table I. ^{1}H NMR assignments for teniposide in deuterochloroform.

chemical shift δ (p.p.m.)	multiplicity	number of protons	assignment (protons at carbon number)
2.89	m	1	3
3.31	t	1	g2
3.42	dd	1	2
∿ 3.45	m	1	g4
3.47	m	1	g5
3.58	t	1	g3
3.69	s	6	OCH_3
3.74	t	1	g6a
4.21	t	1	11'
4.28	dd	1	g6e
4.42	dd	1	11
4.51	d	1	1
4.57	d	1	g1
4.96	d	1	4
5.10	s (br)	1	OH
5.78	s	1	g7
5.94	dd	2	A
6.24	s	2	2',6'
6.49	s	1	8
6.94	s	1	5
6.95	dd	1	t_4
7.15	d	1	t_3
7.30	d	1	t_5
7.81	s	1	4'-OH

Table II. Proton-proton coupling constants for teniposide.

coupled protons	J (Hz)
1,2	5.3
2,3	13.8
3,4	3.2
3,11	8.1
3,11'	10.5
11,11'	10.6
A,A'	~ 1
g_1,g_2	7.8
g_2,g_3	8.5
g_3,g_4	8.5
g_4,g_5	8.1
g_5,g_{6a}	10.1
g_{6a},g_{6e}	10.1
g_5,g_{6e}	4.8
t3,t4	3.3
t4,t5	5.0

Table III. ^{13}C NMR assignments for teniposide.

chemical shift δ (p.p.m.)	assignment (carbon number)
37.1	1
40.5	3
43.1	2
55.8	OCH_3
65.5	g5
67.3	4
68.0	g6
71.9	11
72.5	g3
74.1	g2
80.2	g4
97.8	g1
100.8	A, g7
107.8	2', 6'
109.1	8
109.9	5
125.4	t5
125.8	t3
127.5	4'
129.9	t4
132.6	8''
133.9	4''
139.3	t2
146.3	3', 5', 6, 7
147.9	1'
174.9	13

4.5. Mass Spectrum

The electron impact mass spectrum (EI-MS) of teniposide (Figure 6) was measured with a Kratos MS-80 mass spectrometer. The sample was introduced into the ion source (250 °C) by a direct inlet probe. An electron energy of 70 eV and an ionizing current of 100 µA were used. The base peak in the spectrum is the ion at m/e 382. This fragment results from the loss of OH and the glucopyranosyl moiety (structure b, Scheme II).

The fragment corresponding to the loss of only the glucopyranosyl moiety, at m/e 400 (structure a), has a relatively low abundance (3-5%).

An other fragmentation pathway is the formation of a fragment at m/e 154 corresponding to structure c. Minor fragmentation pathways are the formation of fragments at m/e 246 (structure d) and 201 (structure e), both pathways starting from mass 400 (a) [6].

4.6. Melting Range

The reported melting ranges are:
Teniposide crystallized from ethanol: 246-255°C [5].
Teniposide as obtained from the manufacturer: 237°C, with decomposition [6].

4.7. Differential Scanning Calorimetry

The DSC thermogram for teniposide (Figure 7) was recorded with a Setaram DSC-III, with a scan rate of 3 K/min. The sample size was about 2 mg. The DSC thermogram was recorded in a closed vessel.

Endothermic peaks appear at approximately 194 and 218°C, the integrated endothermic effect being 50.4 J/g. The strong exothermic peak has its maximum at 235°C, and possibly results from decomposition. When the vessel is

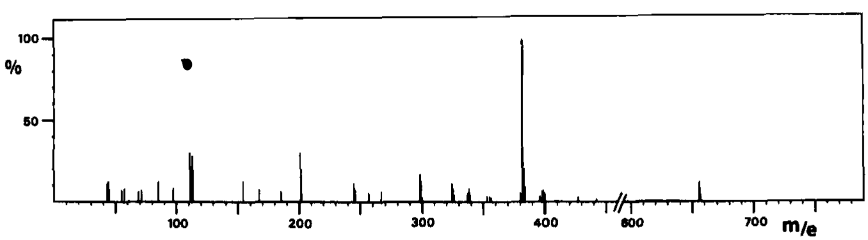
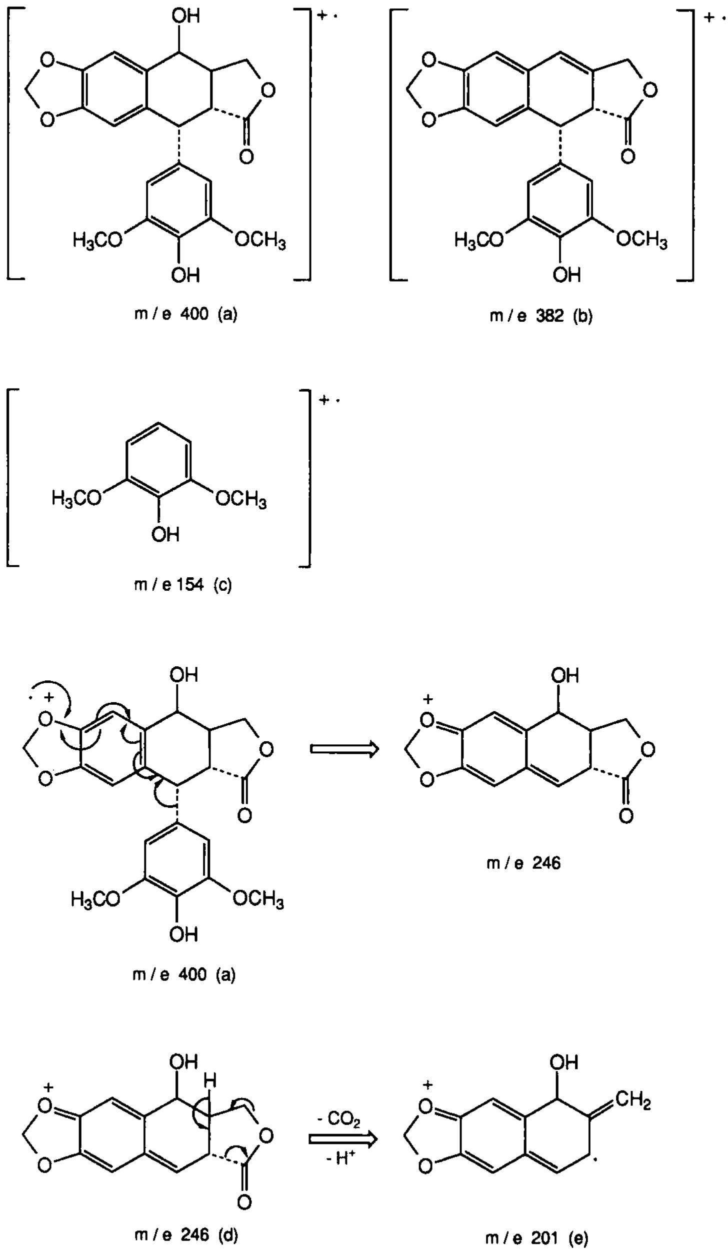


Figure 6. The EI mass spectrum of teniposide.



Scheme II. Fragmentation pathways of teniposide.

cooled down and heated a second time, neither an endothermic nor an exothermic effect is observed, indicating that the material has changed.

4.8. Optical Rotation

The optical rotation $[\alpha]_D^{21}$ of teniposide crystallized from ethanol (c = 0,5 g/v, $CHCl_3$/MeOH, 9:1) was -108.6° [5].

4.9. Dissociation Constant

The pK_a of the C4' phenolic function was determined spectrometrically. Spectra of 0.8 x 10^{-4} M solutions of teniposide in 0.05 M sodium borate buffers containing 5% DMSO (v/v) were recorded with a Shimadzu UV-200 double beam spectrometer. The ionic strength was kept at 0.15 M by addition of KCl. From the inflexion in the plot of the absorbance as a function of pH, a pK_a of 10.13 was found [8].

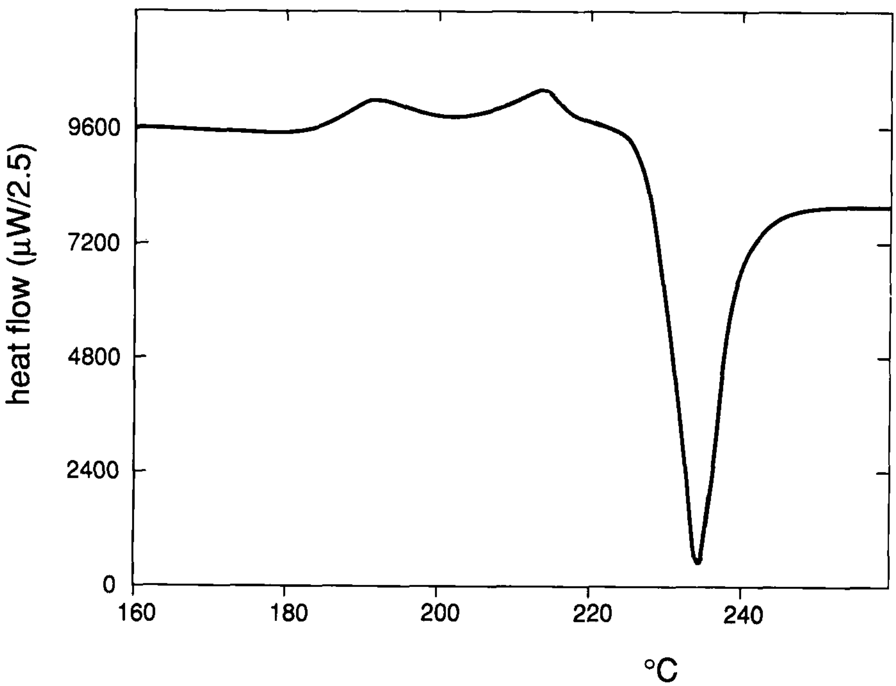


Figure 7. The DSC thermogram of teniposide (exothermic process).

4.10. Electrochemistry

Teniposide contains a 2,6-dimethoxyphenol group (ring E), which can be oxidized chemically or electrochemically. The oxidation mechanism of teniposide was studied in aqueous solutions buffered at different pH values. The cyclic voltammogram of teniposide at pH 7.0 is presented in Figure 8 [8].

The electrochemical oxidation of teniposide in aqueous solutions shows an overall transfer of two electrons. At pH values below 2.5, the oxidation proceeds in one voltammetric, pH-independent oxidation step (1, Figure 9). At pH values above 2.5, the oxidation proceeds in two voltammetric oxidation steps. The transfer of the first electron (3, Figure 9) is reversible and is preceded by a proton transfer (2, Figure 9). The transfer of the second electron (4) results in the formation of an unstable cation which is converted rapidly into the o-quinone of teniposide (5).

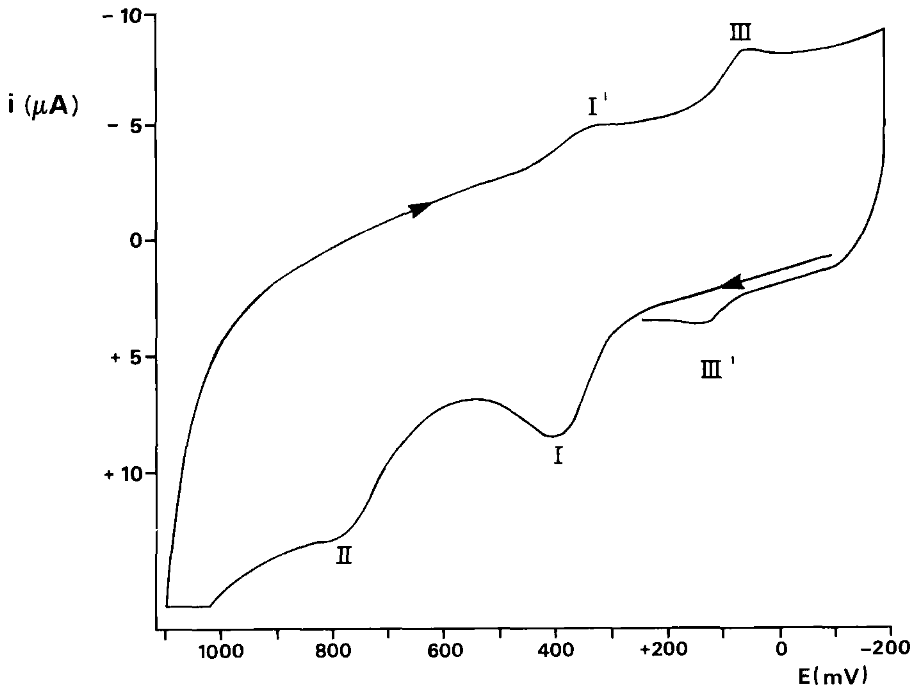


Figure 8. Cyclic voltammogram of 0.125 mM teniposide in 0.1 M phosphate buffer pH 7.0 at a glassy carbon electrode. Scan rate 0.1 V/s. The cyclic voltammogram was recorded from -0.2 V to +1.1 V and back +0.2 V.

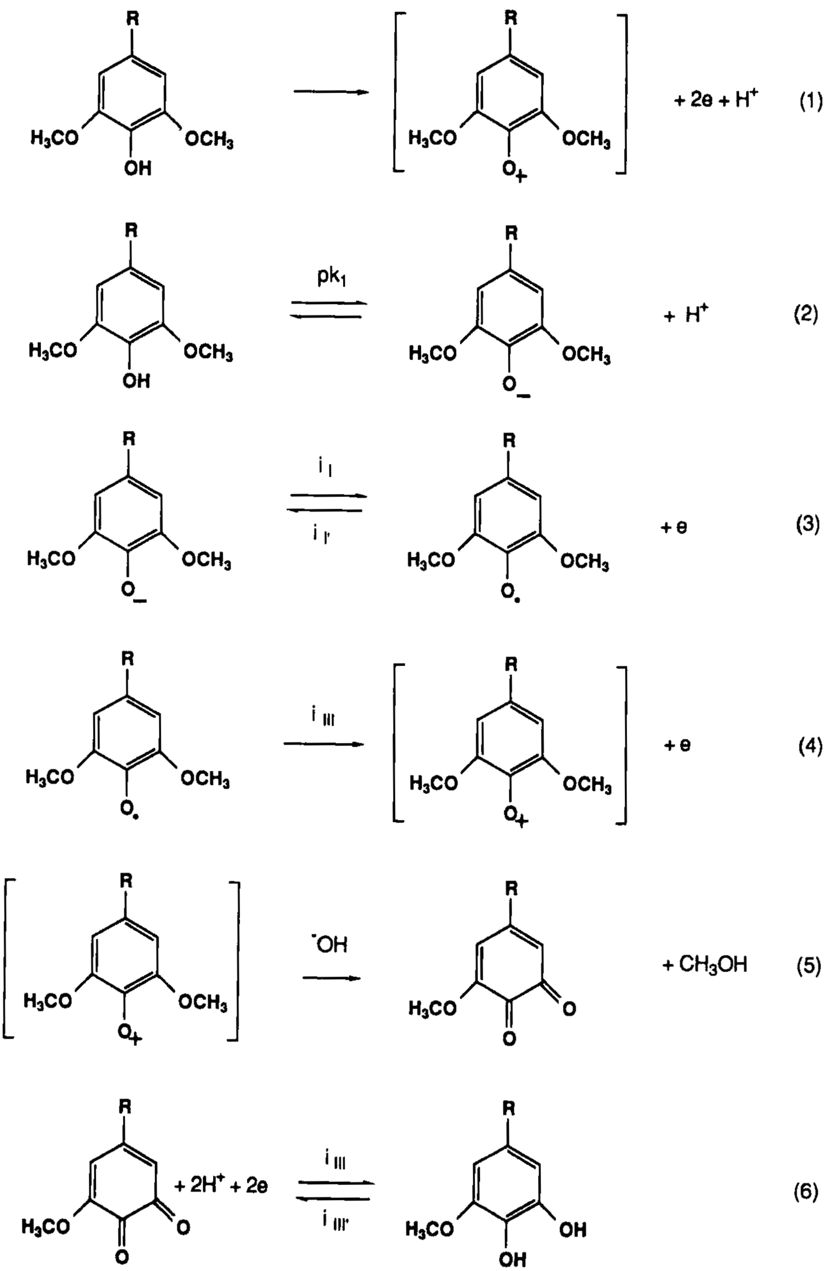


Figure 9. Oxidation mechanism of teniposide.

The o-quinone is adsorbed at the electrode surface, and is reduced in the cathodic scan (i_{III}) to the corresponding hydroquinone. The hydroquinone is oxidized in the second anodic scan ($i_{III'}$). Both the oxidation of the hydroquinone and the reduction of the o-quinone are pH-dependent [8].

5. METHODS OF ANALYSIS

5.1. Thin Layer and Paper Chromatography

Information on thin layer chromatographic systems and on paper chromatography is scanty. Only a few systems (Table IV) have been described [9].

Table IV. Thin layer and paper chromatography of teniposide.

phase	solvent (v/v)	Rf
silicagel	chloroform methanol (21:1)	?
	butanol-glacial acetic acid-water (3:1:1)	?
cellulose paper	butanol-glacial acetic acid-water (3:1:1)	?

5.2. High Performance Liquid Chromatography

Table V presents a few liquid chromatographic methods used for the determination of teniposide in solutions. The unpublished method is used for the determination of the stability of teniposide and was shown to be stability-indicating (see section 6.2).

However, the reversed phase chromatography methods published until now were mainly developed for the analysis of teniposide in biological materials (section 8, Table VI).

Table V. High performance liquid chromatography of teniposide.

column	mobile phase	detection	reference
Lichrosorb RP18 (300x3.9 mm) 10 µm	methanol/20 mM phosphate buffer pH 7 55/45 w/w	dynamic voltammetric detection	10
Novapak Phenyl (75x3.9 mm) 4 µm	methanol/10 mM phosphate buffer pH 7.0 (55/45 w/w)	ECD + 500 mV vs. Ag/AgCl	not published

6. STABILITY AND DEGRADATION

6.1 Stability in Aqueous Solutions

As etoposide, teniposide possesses a strained *trans* lactone ring (Figure 10). This *trans* lactone ring is subject to degradation in both acidic and alkaline media. The degradation of teniposide has been studied in less detail than that of etoposide [11]. However, it is assumed that the degradation reactions are essentially the same. In acidic media the glucopyranosyl moiety is cleaved, yielding 4'-demethylepipodophyllotoxin (the aglycon) (I, Figure 10). The aglycon degrades further to the *trans* hydroxy acid of 4'-demethylepipodophyllotoxin (II).

At pH values >5, the degradation of teniposide occurs through epimerization of the *trans*-fused lactone ring to the *cis*-fused lactone (III). Further degradation results in the formation of the *cis*-hydroxy acid (IV). Conversion of the *trans* lactone ring into the *cis* lactone ring (teniposide → III) at pH > 4 occurs through enolization and subsequent conversion of the enol(V) into *cis*-teniposide (III). The last-mentioned reaction requires proton transfers, which are facilitated by bases such as OH^-, H_2O, or anions of the acid used as a buffer.

6.2. Stability in Plasma

Possible chemical instability of teniposide could cause problems in bioanalysis. Therefore, the stability of

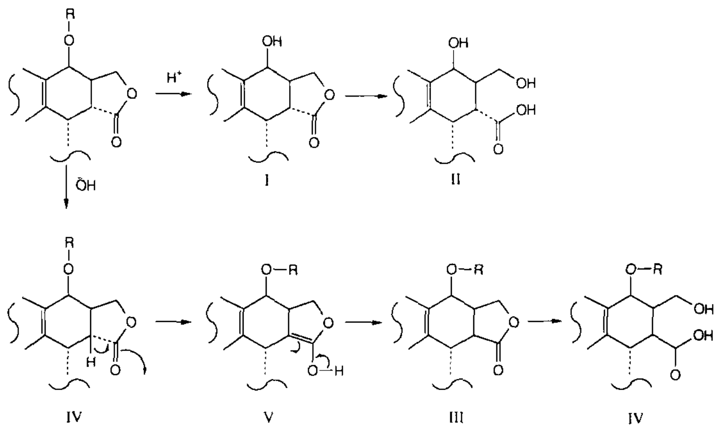


Figure 10. Degradation pathways of teniposide in acidic and alkaline media (R = glucopyranosyl).

teniposide in plasma was studied at 37, 4, and -18°C. A stability-indicating assay was used allowing quantification of teniposide, *cis*-teniposide and 4'-demethylepipodophyllotoxin, after isolation from biological matrices [6]. (See Table V, unpublished results).

When teniposide in plasma is stored at 37°C, a decrease in the concentration is observed after 12 hours. This decrease is caused either by chemical degradation or is due to adsorption to precipitated protein. When plasma samples containing teniposide are stored at 4°C or -18°C, no decrease in the concentration was observed for at least 8 weeks. These studies allow the conclusion that it is not necessary to freeze plasma samples immediately after preparation.

7. PHARMACOLOGY

7.1. Mechanism of Action

Teniposide differs in its biological action from its parent podophyllotoxin, which is a spindle poison. Teniposide does not interact with the microtubule assembly [12,13], but prevents cells from entering mitosis. In contrast, the precursor podophyllotoxin arrests cells in the metaphase. Teniposide accumulates cells in the G2 phase.

Cells treated with teniposide show a rapid decrease of the mitotic index, with a simultaneous reduction of cell proliferation.

Teniposide has been shown to induce double strand breaks and single strand breaks in DNA in intact cells and in nuclei, but not in purified DNA. The DNA degradation is dose- and temperature-dependent, and reversible after removal of the drug [12-15].

Teniposide is believed to be activated in the cell nucleus by oxidation of the phenolic group to reactive intermediates [16]. Interaction of these intermediates with DNA could also result in DNA damage.

Studies indicate that type II topoisomerase is probably the intracellular target in the DNA strand-breaking property of teniposide [16-19].

Teniposide inhibits the cellular uptake of thymidine, uridine, adenosine, and guanosine [14]. The binding of teniposide to cell constituents is seven to ten times as high as that of etoposide [20], and as a result, the uptake of teniposide in the cell is ten times higher. Teniposide proved to be more active in the L1210 system than etoposide [15].

7.2. Pharmacokinetics

Up to now, teniposide is only administered intravenously. The pharmacokinetics of teniposide after intravenous infusion is described by an open two-compartment model [21-26] or by an open three-compartment model [9,27]. No difference is observed in the disposition of teniposide administered at low or at high doses [27,28].

The elimination half-life time after intravenous administration is about 10 hours [28], which is about two times longer than is found for etoposide.

Figure 11 presents possible metabolic pathways of teniposide. Although teniposide is mainly cleared from the body by metabolic activity, little information is available. Most of the metabolic studies on the epipodophyllotoxins have been performed with etoposide, a congener of teniposide.

Pathway A: Small amounts of *cis*-teniposide have been detected in urine and plasma by reversed phase chromatographic methods. *In vitro*, this conversion is more pronounced at elevated temperatures and at higher pH values [25,31].

Pathway B: The formation of the *cis* or *trans*-hydroxy acid of teniposide is probably a minor metabolic pathway.

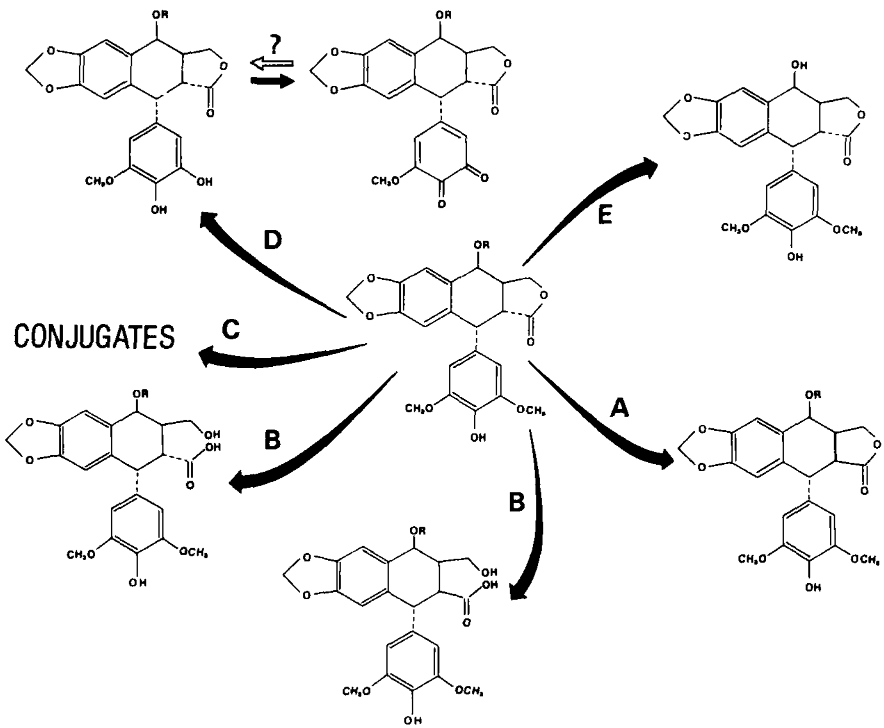


Figure 11. Possible metabolism of teniposide (R=glucopyranosyl).

Only one research group found an indication for the presence of this metabolite in urine [31].

Pathway C and D: Up to now, these pathways were found to be important for etoposide only.

Pathway E: An indication for relatively large amounts of conjugated (glucuronidated) 4'-demethylepipodophyllotoxin was found in patients [21] receiving teniposide, on prolonged intravenous infusion. However, the identity of this metabolite was not confirmed by, e.g., mass spectrometry or NMR.

7.3. Clinical Activity

Information on the clinical pharmacology was reviewed by O'Dryer et al. [1]. Teniposide is active against leukaemias, lymphoma, and neuroblastoma.

7.4. Clinical Toxicity

The dose-limiting toxicity of teniposide was shown to be dose-related myelosuppression. Another toxic effect is hypotension, which is associated with rapid infusion of the drug. Other minor adverse reactions are vomiting and alopecia [1].

8. ANALYSIS OF TENIPOSIDE AND METABOLITES IN BIOLOGICAL MATRICES

8.1. Teniposide

The first pharmacokinetic and metabolic studies with teniposide were performed with a ^{3}H-labelled drug [9,29]. In these studies, the parent compound was separated from non-extractable metabolites and from the biological matrices by extraction with chloroform [9,29]. The purity was checked by means of a TLC system [section 5.1].

Apart from the HPLC methods, a radioimmunoassay for teniposide was developed. The sensitivity of this method proved to be comparable to an HPLC method [30]. The method is not specific for the parent compound: cross reactions with etoposide, metabolites, and degradation products are observed.

The methods published for the bioanalysis of teniposide are carried out with reversed phase HPLC.

Table VI summarizes the reversed phase liquid chromatographic methods for the analysis of teniposide in biological fluids. The methods mentioned in this table are also suitable for the analysis of etoposide. The published methods are mostly dealing with the analysis of plasma; a few are also used for the analysis in urine and cerebrospinal fluid (CSF). Teniposide is mainly isolated by extraction with an organic solvent prior to the HPLC analysis. Ethyl acetate [31], chloroform [6,32,33,34], and 1,2-dichloroethane [6] are used. A few methods apply column extraction [35,36], allowing rapid analysis of the drug.

The described extraction methods are likely to be also suitable for the analysis of the neutral metabolites 4'-demethylepipodophyllotoxin and *cis*-teniposide. Two chromatographic systems have been reported for the separation of *cis*- and *trans*-teniposide [6,31].

Table VI. Published HPLC methods for the analysis of teniposide in biological fluids.

matrix	sample pretreatment	column	detection	determination limit	reference
plasma	chloroform extraction	Bondapak C_{18}, 10 µm	UV 254 nm	500 ng/ml	32
plasma	chloroform, extraction	Bondapak C_{18}, 10 µm	fluorescence 215/328 nm	50 ng/ml	33
urine plasma CSF	ethyl acetate extraction after addition of $(NH_4)_2SO_4$	Bondapak Phenyl, 10 µm	ECD* + 0.85 V vs. Ag/AgCl	20 ng/ml	31
plasma urine	preconcentration on PRP.1, post-column extraction with 1,2-dichloroethane	Lichrosorb RP 18, 10 µm	fluorescence 230/328 nm	8 ng/ml 30 ng/ml	35
plasma	1,2-dichloroethane extraction	Bondapak Phenyl, 10 µm	ECD + 0.50 V vs. Ag/AgCl	5 ng/ml	6
plasma	solid-phase extraction C-18 Bond Elut	ODS Hypersil 5 µm	ECD + 0.9 V	500 ng/ml	36
plasma urine	chloroform extraction	Lichrosorb RP 18, 10 µm	UV 280 nm 254 nm	50 ng/ml 70 ng/ml	34
plasma	injection of plasma after addition of 10% SDS	Chromspher C_{18}, 5 µm with Chromspher C_{18}, 40 µm pre-column (40°C)	ECD + 0.65 V vs. Ag/AgCl	500 ng	38
plasma	injection of plasma after addition of 10% SDS; on-line pre-column extraction	Bondapak Phenyl, 10 µm with Chromspher C_{18}, 40 µm pre-column	ECD + 0.50 V vs. Ag/AgCl	10 ng/ml	39

* ECD = electrochemical detection

Recently, a flow injection method (FIA) was developed for the determination of teniposide in plasma. In this method, teniposide is converted by on-line electrochemical oxidation into the o-quinone. Detection takes place at 365 nm which enables the determination of 1 µg teniposide per ml plasma. Prior to injection, teniposide is extracted with 1,2-dichloroethane [37].

A method using micellar chromatography [38] was developed recently. This method allows the direct injection of (20 µl) plasma samples. A detection limit of 0.5 µg/ml is obtained. In a second method with micelles, sodium dodecyl sulfate (SDS) is added to plasma prior to pre-column extraction; the extraction is followed by reversed phase liquid chromatography [39]. The last-mentioned method has a detection limit of 10 ng per ml plasma.

8.2. Teniposide Metabolites

No specific methods have been developed for the analysis of metabolites in urine or plasma. According to the proposed metabolic pathway (7.2.), several metabolites are possible. These metabolites differ significantly in their physico-chemical properties. The metabolites with intact lactone rings can be separated by traditional reversed phase liquid chromatagraphy on a phenyl-bonded phase after liquid-liquid extraction. For the chromatography of hydrophilic metabolites, e.g., hydroxy acids and glucuronides, a different approach is necessary.

ACKNOWLEDGEMENT

The authors are grateful to Mr. D. Seykens, Department of Organic Chemistry, University of Utrecht, The Netherlands for running the NMR spectra and providing relevant ^{13}C reference data.

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Terbutaline Sulfate

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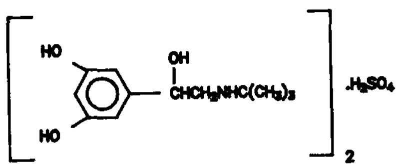
Terbutaline Sulfate

1. Description

1.1 Introduction

Terbutaline sulfate is a synthetic β_2-adrenoceptor that is used as a bronchodilator in the treatment of bronchial asthma.

1.2 Formula, Name, Formula Weight



Terbutaline Sulfate

Formula Weight: 548.658 $C_{24}H_{40}N_2O_{10}S$

Terbutaline sulfate has been described by the following chemical names:

(i) 5-[2-[(1,1-Dimethylethyl)amino]-1-hydroxy_ ethyl]-1,3-benzenediol sulfate (2:1 salt)

(ii) α-[(tert-Butylamino)methyl]-3,5-dihydroxy-benzyl alcohol sulfate (2:1 salt)

(iii) 1-(3,5-Dihydroxyphenyl)-2-(tert-butylamino)-ethanol sulfate (2:1 salt)

1.3 Appearance, Color, Odor

Terbutaline sulfate is a white to gray-white crystalline powder, odorless or with a faint odor of acetic acid.

2. Physical Properties

2.1 Ultraviolet Spectroscopy

The ultraviolet absorption spectrum of terbutaline sulfate in 0.1N hydrochloric acid (Figure 1)

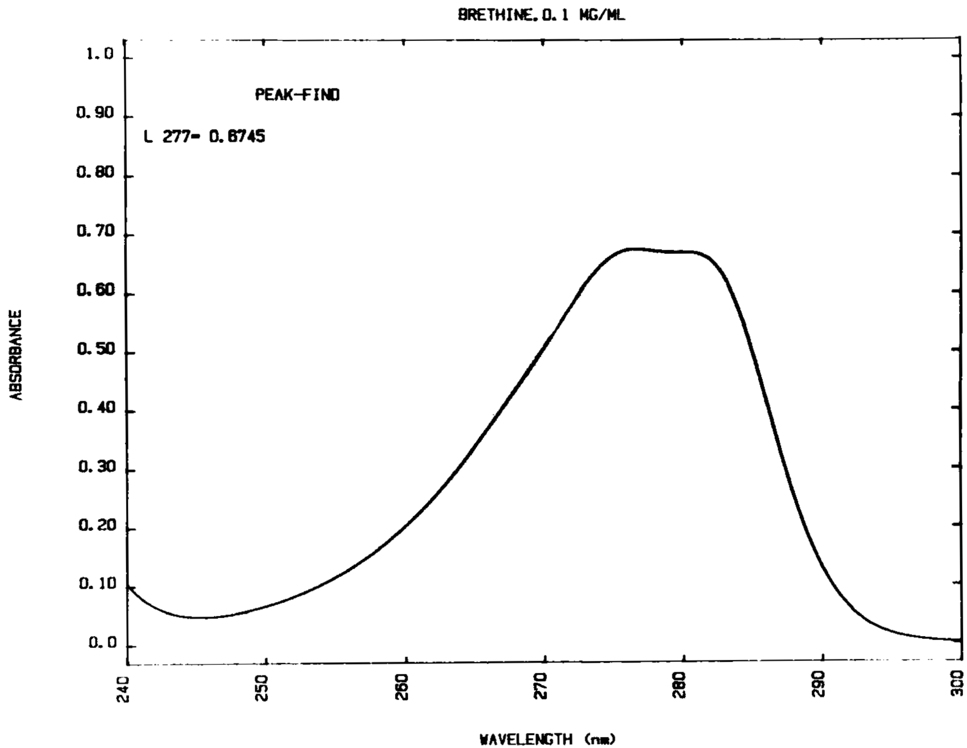


Figure 1 Ultraviolet Spectrum of Terbutaline Sulfate (S-4-90-4) in 0.1 N HCl

exhibits a λmax at 276nm and yields an A(1%,1cm) value of 67.6. This absorption arises from the $\pi \rightarrow \pi^*$ transition of the electrons in the phenyl ring (aromatic) (1).

2.2 Infrared Spectroscopy

The infrared absorption spectrum of terbutaline sulfate in a KBr pellet (Figure 2) exhibits the following bands which are consistent with its structure (1):

Wavenumber (cm^{-1})	Assignment
3330	OH stretch
3050	aromatic CH stretch
2970	methyl asymmetric stretch
2920	methylene asymmetric stretch
2720-2900,2660,2500	secondary amine salt stretch
1610,1485	aromatic ring stretch
1455	methylene scissoring/-asymmetric bend
1400,1380	t-butyl symmetric bend
1240,1210	t-butyl characteristic absorbances
1200	phenolic C-O stretch
1065	secondary alcohol C-O stretch
850, 690	1,3,5 trisubstituted benzene, out-of-plane bend

2.3 Nuclear Magnetic Resonance Spectroscopy

The NMR spectrum of terbutaline sulfate in deuterium oxide (Figure 3) is consistent with its structure (1):

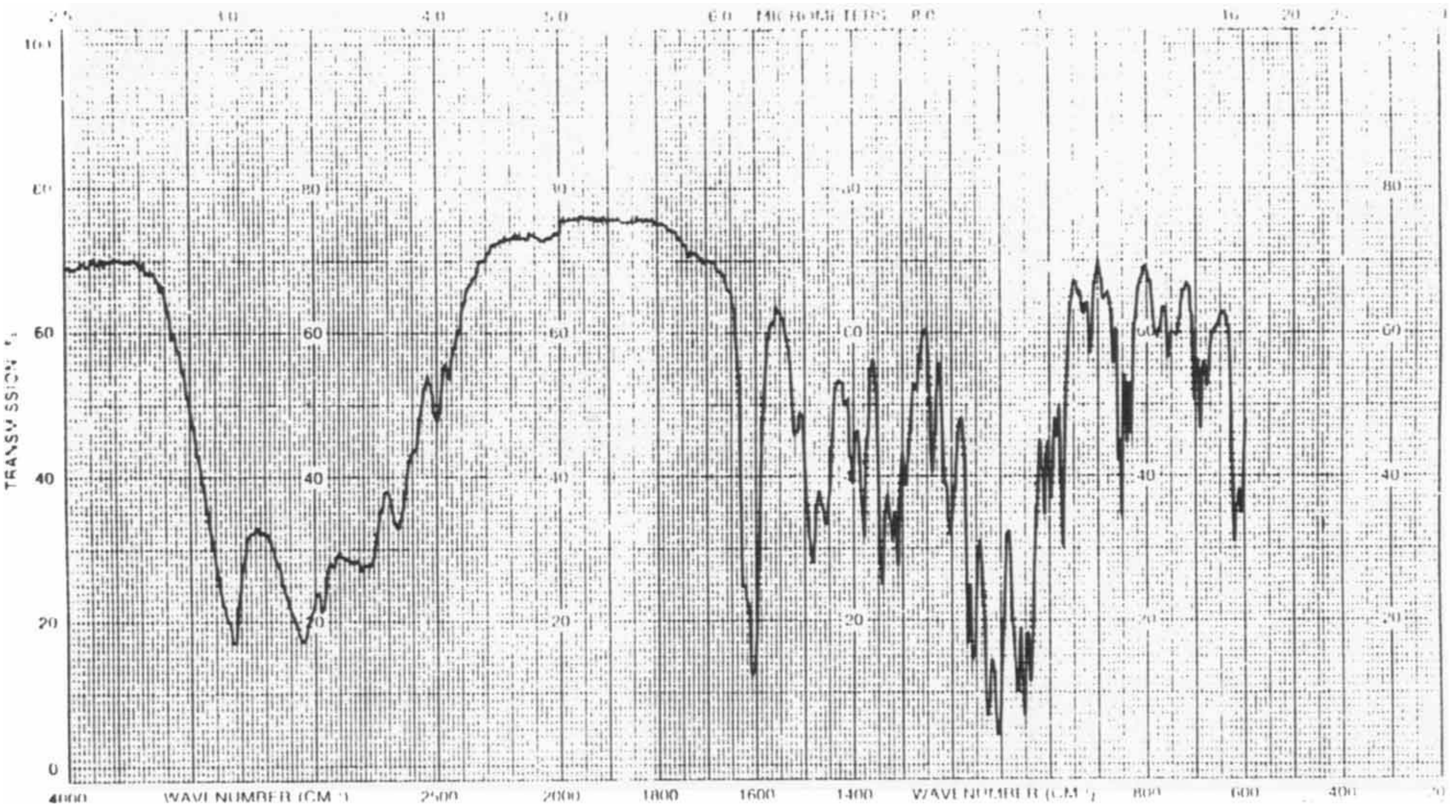


Figure 2 Infrared Spectrum of Terbutaline Sulfate (S-4-90-4)

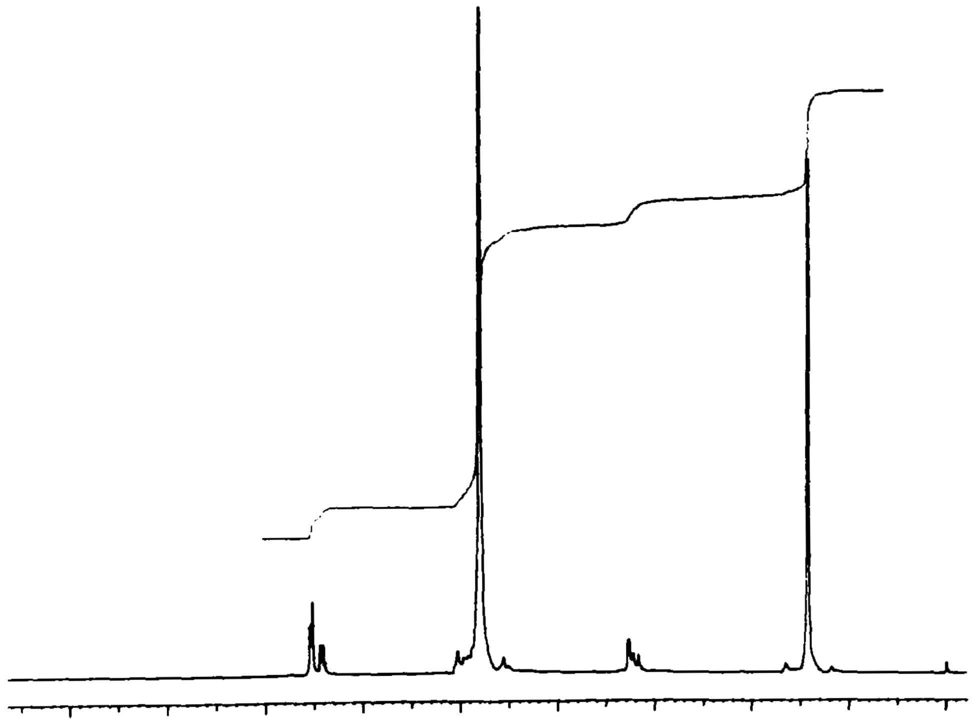
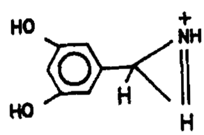
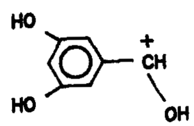


Figure 3 NMR Spectrum of Terbutaline Sulfate (S-4-90-4) in D_2O

Chemical Shift (ppm)	Multiplicity	Relative Integral of Protons	Assigment
1.45	Singlet	9	Methyl Protons
3.1 - 3.4	Triplet	2	Methylene Protons
4.7 - 5.1	Multiplet	---	Methine Proton + Exchangeables
6.35 - 6.7	Multiplet	3	Aromatic Protons

2.4 Mass Spectrometry

The mass spectrum (Figure 4) is compatible with the indicated structure of terbutaline sulfate and shows the following fragmentation pattern (1).

m/e	Structure
225	M^+
210	$M-CH_3$
207	$M-H_2O$
192	$M-(CH_3+H_2O)$
150	
139	

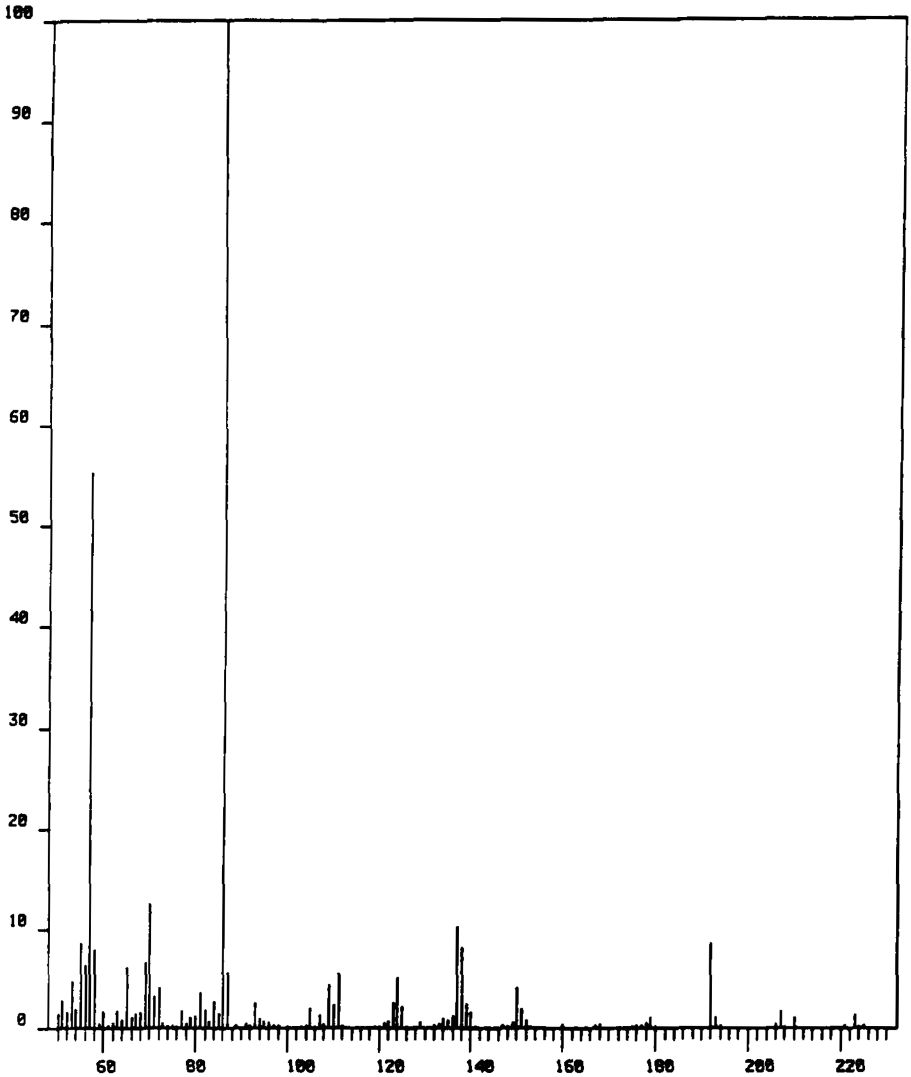
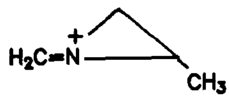


Figure 4 Mass Spectrum of Terbutaline Sulfate (CDF 2037)

111	139-CO
86	$H_2C{=}\overset{\oplus}{N}{-}C(CH_3)_3$ H H
70	
57	CH_3 | $\oplus C{-}CH_3$ | CH_3

2.5 Circular Dichroism

Commercially sold terbutaline sulfate is racemic; no circular dichroism is observed.

2.6 Differential Scanning Calorimetry

No reliable purity value can be determined since terbutaline sulfate melts with decomposition.

2.7 Melting Range

As noted above, terbutaline sulfate decomposes upon melting. Melting points determined by DSC for batches of terbutaline sulfate identified as crystal form A range from 264°C to 271°C. For batches identified as crystal form B, these values range from 258°C to 260°C (1).

2.8 Thermogravimetry

Thermogravimetric analysis generally gives a weight loss of less than 0.5% between RT and 220°C (1).

2.9 X-ray Powder Diffraction

Two crystal forms have been characterized by x-ray powder diffraction patterns (1). The A form shows lines at 8.5, 9.4, 10.2, 11.9, 13.0, 15.2, 16.8, 17.3, 18.0, 19.6, 21.2, 22.0, 22.6, 24.1, 26.0 and 27.0 degrees 2θ. The B form shows lines at 6.9, 8.1, 9.2, 10.6, 12.4, 13.1, 13.9, 16.1, 16.6, 17.9, 18.6, 19.0, 19.8, 21.0, 23.2, 24.3, 25.4 and 27.2 degrees 2θ (Figure 5).

2.10 Dissociation Constant

The following pKa values have been reported for terbutaline sulfate: 8.8, 10.1 and 11.2. The 10.1 value can be assigned to the amino group. The other two pKa values (8.8 and 11.2) may be attributed to the aromatic hydroxyl groups of this compound. (1)

2.11 Solubility

The equilibrium solubility values in various solvents, at 25°C, are given in Table I (1).

TABLE I

Solubility of Terbutaline Sulfate

Solvent	mg/mL
Water	>20
0.1N HCl	>20

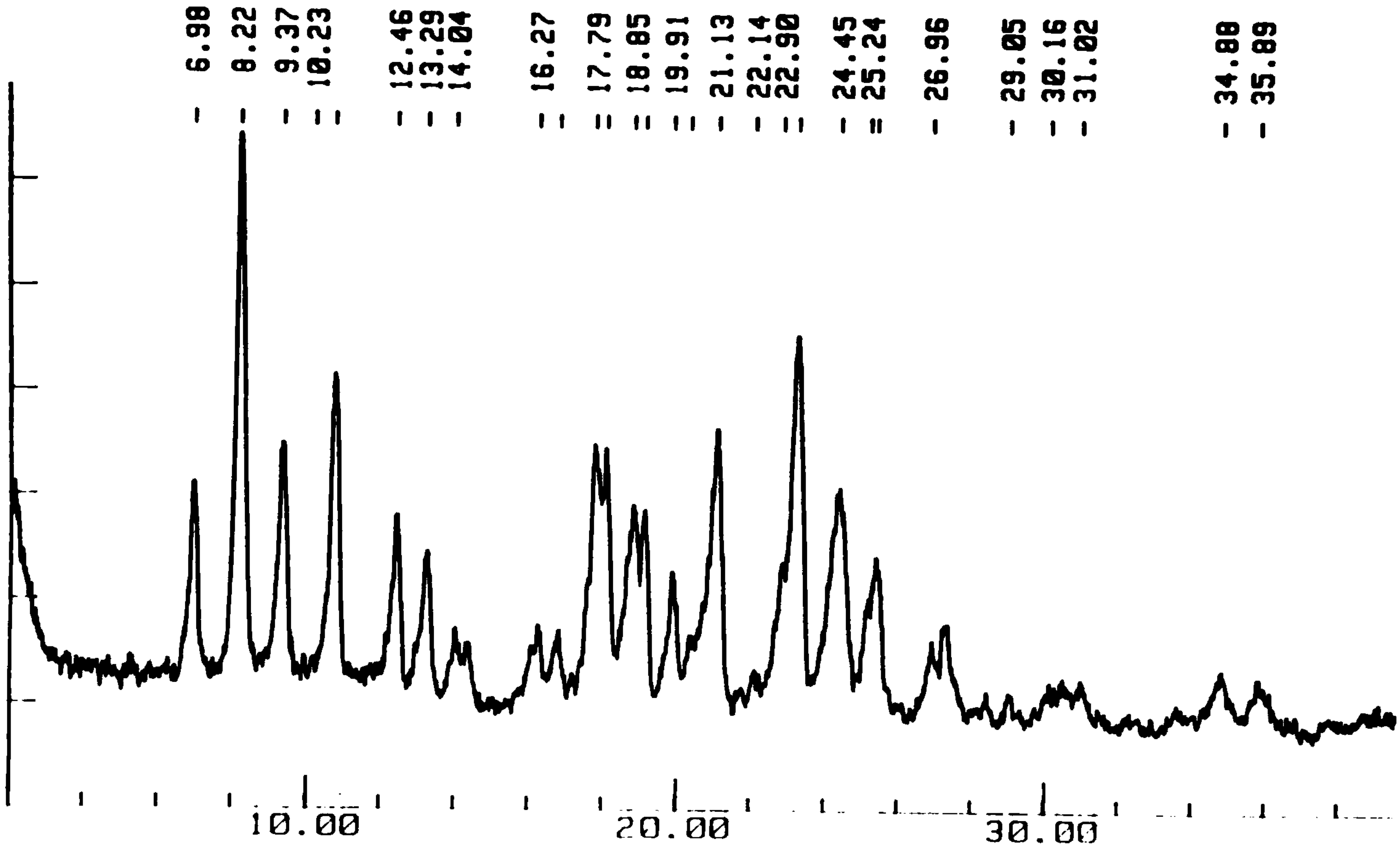


Figure 5 X-ray Diffraction of B form of Terbutaline Sulfate (S-4-90-4)

Table I

Solubility of Terbutaline Sulfate
(cont'd)

0.1N NaOH	>20
Ethanol	1.2
10% Ethanol	>20
Methanol	2.7

2.12 Water Absorption

A 0.2% gain in water content has been observed with the samples stored at 25°C/75% R.H. for 14 days.

2.13 Distribution Coefficient

Distribution coefficients in various organic phases vs. aqueous phase at room temperature are given in Table II (1).

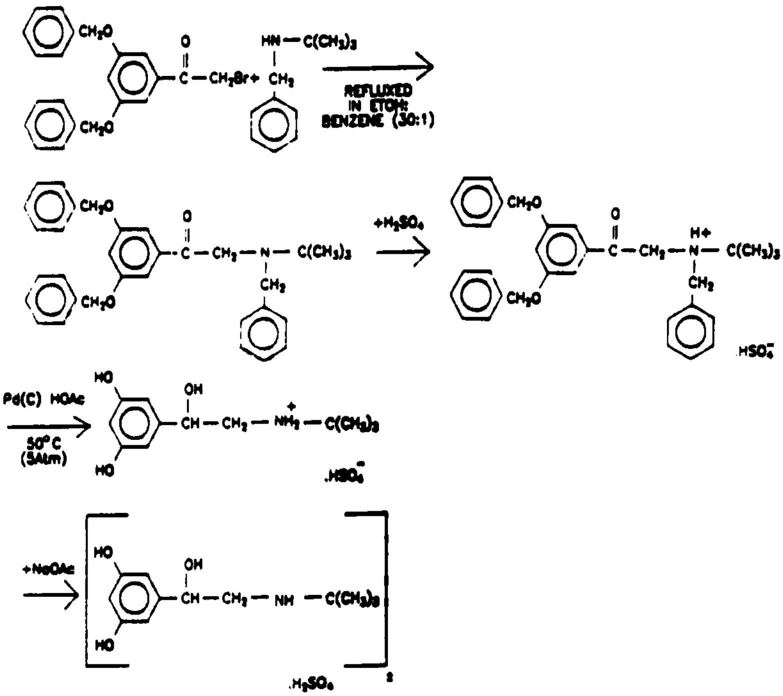
TABLE II

Distribution Coefficients of Terbutaline Sulfate

Organic Phase	Aqueous Phase	$K = \frac{C_{org.}}{C_{aq.}}$
Chloroform	0.1N HCl	0.0
Ether	0.1N HCl	0.0
Isooctane	0.1N HCl	0.0
Chloroform	0.1N NaOH	0.0

3. Synthesis

The synthesis for terbutaline sulfate given below is based on a U.S. patent issued to Wetterlin, et al (2).



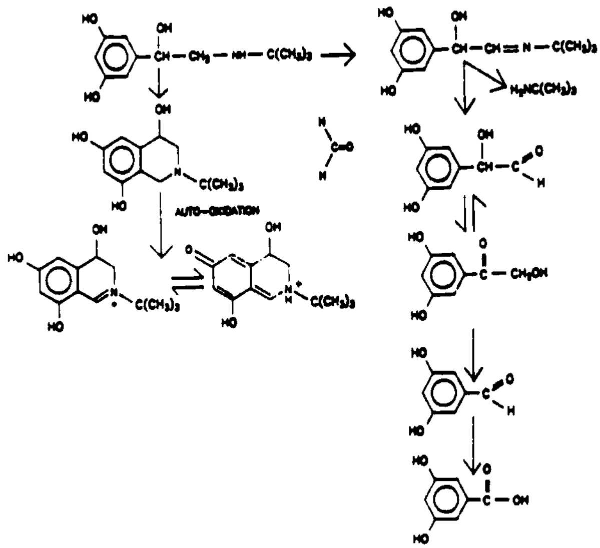
4. Stability

4.1 Solid State Stability

Terbutaline sulfate is a stable compound under normal storage conditions. No change in chromatographic impurities was detected after three years storage at room temperature (1).

4.2 Solution Stability

Discoloration of aqueous solutions of terbutaline sulfate is caused by oxidation of terbutaline and is enhanced by low pH and by ultratrace levels (ppb) of metals in the presence of oxygen. HPLC analysis of degraded terbutaline sulfate solutions suggest that oxidative degradation is favored (3,4).



5. Pharmacokinetics, Metabolism and Activity

Distribution data (Section 2.13) suggests terbutaline sulfate is hydrophilic.

5.1 Absorption/Excretion/Elimination

The structure of terbutaline sulfate prevents it from being metabolized by catechol-O-methyl transferase or monoamine oxidase (5,6). The principle pathway of metabolism is conjugation with sulfuric

acid or gulcuronic acid. However, there are species differences and the extent of metabolism depends on the route of administration.

Following oral administration, approximately 50% of the dose is excreted unchanged in the feces. In a 24 hour collection period, 5.7% of the dose was recovered unchanged in the urine, while 16.8% was recovered as conjugated terbutaline. In a 72 hour collection period 92% of the dose was recovered with 52% in the feces and 6% in the urine as unchanged terbutaline (5). Oral absorption capacity ranges have been estimated with a range of 25-80%, this creates a range in the extent of bioavailability of 7-26%, the decrease in the percentages being due to a high first pass metabolism . The terminal half-life in healthy subjects is approximately 17 hours (7). The biological half-life is 3.6 hours (8). Although absorption from the gastrointestinal tract is incomplete, peak plasma levels of unchanged terbutaline reach approximately 5 ng/mL (8,9), which apparently is sufficient to produce effective changes in pulmonary function.

The pattern of metabolism following oral inhalation of terbutaline from a metered dose dispenser is similar to the pattern observed following oral ingestion. This is probably because approximately 90 percent of the inhaled does is swallowed (10,11).

The patterns of metabolism and excretion following intravenous and subcutaneous administration are essentially identical. Only 2 percent of the drug can be recovered in the feces following parenteral administration (6,9). A significant amount of unchanged terbutaline is excreted in the urine following intravenous or subcutaneous administration. Approximately 60 percent of the administered

dose is recovered as unchanged terbutaline in the urine (6,12). The presence of an unidentified metabolite in the urine, which represented approximately 15 percent of the administered dose, was reported by Davies et al (6). The high percentage of conjugated terbutaline following oral administration suggest significant sulfate conjugation took place in the gut wall or during the first pass through the liver.

After a single intravenous dose, plasma concentrations of terbutaline decline in a multi-exponential fashion (13). The terminal half-life is roughly 17 hours with a two-fold variation between subjects. Intravenously administered terbutaline is excreted mainly in the urine with approximately 60% being recovered as the unchanged drug and between 4 and 19% being recovered as the sulfate conjugate. No other metabolites have been identified. Estimates for total body clearance range from 165 to 170 mL/min. Volumes of distribution at steady-state range from 83 to 140 L.

No statistically significant difference was observed between the serum concentration time profiles for the subcutaneous dose in the healthy volunteer and patient groups. Serum concentrations increased to achieve a peak concentration of 7.2 (± 1.7) ng/mL at 0.43 (± 0.13) hours after dosing. As in the case of the healthy volunteers the subsequent decline from the peak was biphasic. The availability of the drug after oral dosing ranged from 7.0 to 14.0% (mean ± S.D. = 10.0 ± 3.0%) in the 8 asthmatic patients.

In a series of pharmacokinetics studies after oral and intravenous administration of tritium-labelled drug, unchanged terbutaline accounted for less than 15% of the total radioactivity in plasma following oral administration (14). However, after acid-hydrolysis unchanged

terbutaline accounted for 90% of the radioactivity. In contrast, unchanged terbutaline accounted for more than 85% of the total radioactivity over the first 60 minutes after intravenous dosing. These results suggests extensive conjugation of terbutaline before the drug reaches the systemic circulation. The site of pre-systemic conjugation has not been established.

The pharmacokinetics of terbutaline has been measured in two studies after oral and subcutaneous administration of the drug. Maximum concentrations of terbutaline (mean: 3.2 ± 0.4 ng/mL) were achieved at 2 - 4 hours after oral dosing. After subcutaneous dosing maximum levels of 6.9 ± 0.5 ng/mL were observed at 30 minutes. Approximately 30% of the dose was excreted by glomerular filtration in 12 hours and 40% in 72 hours. No obvious differences were detected in the plasma concentration-time profiles for healthy volunteers and patients.

5.2 Drug Binding

Terbutaline plasma protein binding is low, 14 - 25%. In contrast, binding to erythrocytes is more pronounced producing erythrocyte:plasma concentration ratios of 2.0 to 2.6.

5.3 Pharmacodynamics

The relationships between plasma terbutaline concentrations after oral and subcutaneous dosing and pulmonary effects have been investigated in two studies involving 10 and 8 asthmatic patients, respectively. In both studies serum concentrations of the drug were correlated with the forced expiration volume in 1 second. Maximum pulmonary effects occurred at 30 - 60 minutes after the subcutaneous dose and at 2 - 4 hours after the oral

dose. These effects coincided with peak concentrations of terbutaline and c-AMP in plasma. No significant effects on heart rate and blood pressure were noted in one of the studies and the increases in tremor did not appear to parallel either the serum level or bronchodilating effect.

Linear regression analysis of pulmonary responses in children with chronic childhood asthma also showed a statistically significant relationship between the plasma concentration of the drug and its effect on forced expiration volume in 1 second and peak expiratory flow rate (PEFR) within patients. Pharmacodynamic responses increased with ascending doses.

In dogs, the metabolism does not depend on the route of administration (6,15). Very little sulfate conjugate was detected in the urine following intravenous, intraduodenal, or intragastric administration.

In rats, the glucuronide conjugate appeared as the only urinary metabolite (15,16) and the amount of that metabolite formed was not influenced by the route of administration.

5.4 Activity of Enantiomers

The (-) isomer of terbutaline has been found to be 200 times more potent than the (+) isomer for the β_2 receptors (17).

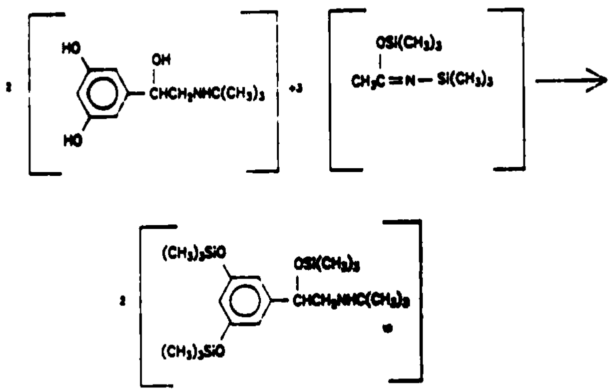
6. Analytical Methodology

6.1 Titrimetry

Terbutaline sulfate active ingredient can be titrated in acetic acid:acetonitrile (1:1) with perchloric acid in acetic acid (18).

6.2 Gas Chromatography

Terbutaline sulfate can be analyzed by gas chromatography by first converting the terbutaline to its tris-trimethyl silyl ether and then chromatographing on 3% OV-17 on an appropriate neutralized, silanized diatomaceous earth support. Column temperatures in the range of 150°C to 190°C have been used. The derivatization scheme follows (19):



6.3 High Pressure Liquid Chromatography

Terbutaline sulfate can be analyzed by reversed-phase and by ion-pairing high pressure liquid chromatography. Several different systems have been used.

Terbutaline sulfate in intravenous solutions has been analyzed by chromatographing on a μBondapak C_{18} column using a mobile phase consisting of 35% methanol (V/V) in an aqueous solution 0.35M in acetic acid and 0.005M in sodium heptanesulfonate (20). In blood, it has been analyzed by chromatographing on a μBondapak C_{18} column using a mobile phase consisting of 5% acetonitrile in a pH 4.0 sodium acetate buffer (21).

An automated method based on liquid chromatography has been utilized for the determination of terbutaline in human plasma in the range of 5 - 50 pmole·mL^{-1} (22). The necessary sensitivity and selectivity was obtained by using electrochemical detection and a microprocessor-controlled column switching system. A combination of three columns was used: a C_8 type for pre-separation, a C_{18} type for trapping and, for final separation, a strongly acidic ion exchanger. The accuracy of the method was examined by comparison with a method based on gas chromatography - mass spectrometry. The overall precision was ± 3.5% and ± 2.2%, respectively at 5 and 50 pmole·mL^{-1}. The total absolute recovery for terbutaline and internal standard at the above concentration levels were in the range 85 - 106%.

A liquid chromatographic method with electrochemical detection (LC-EC) has been found useful for the quantitative analysis of terbutaline in the range 5-50 pmole·mL^{-1} of human plasma (23). Terbutaline is isolated from plasma on an ion-exchange column and the eluate is concentrated on a hydrophobic precolumn on-line in the chromatographic system. The precolumn is then back-flushed for further separation onto a hydrophobic analytical column. The mobile phase is a methanol-aqueous buffer to which sodium perchlorate is added to achieve resolution from interfering peaks. A glassy carbon electrode is used for detection. Comparison has been made with gas chromatography-mass spectrometry (GC-MS) to examine the accuracy of the method.

A commercially available chiral stationary phase containing α_1-acid glycoprotein on silica (Enantio-Pac, LKB) was used for the resolution of enantiomers of terbutaline (24). The mobile phase contained 0.003 $\underline{M}$ tetrapropyl-ammonium bromide adjusted to pH 7.0. Terbutaline enantiomers in

biological samples have been analyzed on a cyclodextrin column (25).

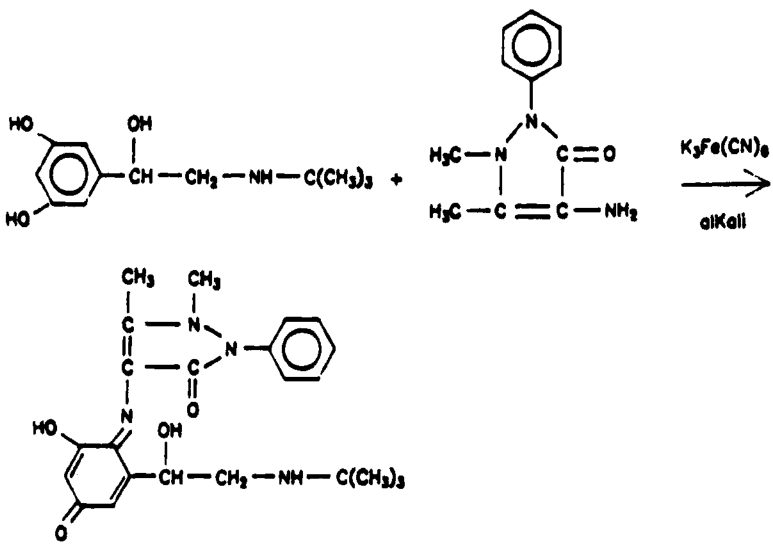
Dosage forms of terbutaline sulfate have been analyzed by chromatographing on a μBondapak phenyl column using a mobile phase consisting of 8% methanol (V/V) in 0.02M pH 3.6 potassium phosphate buffer (26).

6.4. Thin Layer Chromatography

Terbutaline sulfate has been chromatographed on a silica gel plate with a TLC system using a developing solvent consisting of isopropyl alcohol: cyclohexane:formic acid (13:5:1). Detection is accomplished by using 4-aminoanti-pyrine and potassium ferricyanide spray reagents (18).

6.5 Colorimetry

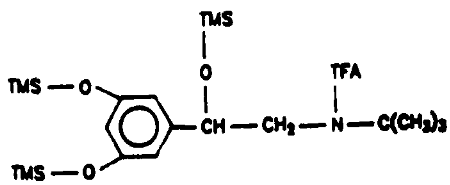
A method based on the following reaction of terbutaline sulfate with 4-aminoantipyrine in the presence of potassium ferricyanide can be used for analysis of terbutaline sulfate dosage forms (18).



6.6 Mass Fragmentography

A simple and sensitive method for the determination of terbutaline in serum and urine has been developed (27). A mass spectrometer in the multiple ion detection mode was used as a gas chromatographic detector. Levels were monitored after oral and subcutaneous administration of the drug. The sensitivity is 1 ng/mL using 1 mL of serum.

A mass fragmentographic method for determination of terbutaline sulfate in biological fluids has been described (19). The mixed TMS-TFA derivative is chromatographed and the m/e 355 ion is monitored. Measurement is possible to 0.3 ng/mL in human plasma.



m/e=355

7. Toxicological Studies

Intravenous toxicological testing in male rats yields an LD_{50} of 48.41 mg/kg ± 1.63 (1).

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TERFENADINE

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CONTENTS

5 - METHODS OF ANALYSIS

6 - STABILITY

7 - PHARMACOKINETICS

1. DESCRIPTION

1.1. Nomenclature

1.1.1. Chemical names

1.1.1.1 - 1-piperidinebutanol, alpha-[4- (1, 1-dimethyl ethyl)phenyl] -4(hydroxydiphenyl methyl)-.
1.1.1.2 - Alpha-[4-(1,1-dimethyl ethyl) phenyl] - 4- (hydroxy diphenyl methyl) - 1-piperidine butanol.
1.1.1.3 - 1-(4(1, 1-dimethyl ethyl) phenyl - 4- (4-hydroxydiphenyl) methyl piperidine) butan-1-ol.
1.1.1.4 - 1(4-Tertbutyl phenyl) - 4- 4- (∝-hydroxybenzhydryl) piperidino butanol.

1.1.2. Generic name

Terfenadine.

1.1.3. Registry number

Chemical Abstract CAS number 50679-08-8.

1.1.4. Wiswesser line notation

T6NIJ A3YQR DX1 of 1 and 1 and DXQR and R.

1.2. Formulae

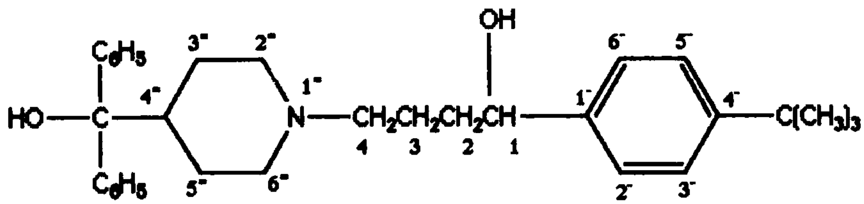
1.2.1. Emperical formula

$C_{32}H_{41}NO_2$.

1.2.2. Molecular weight

471.69.

1.2.3. Structural formula

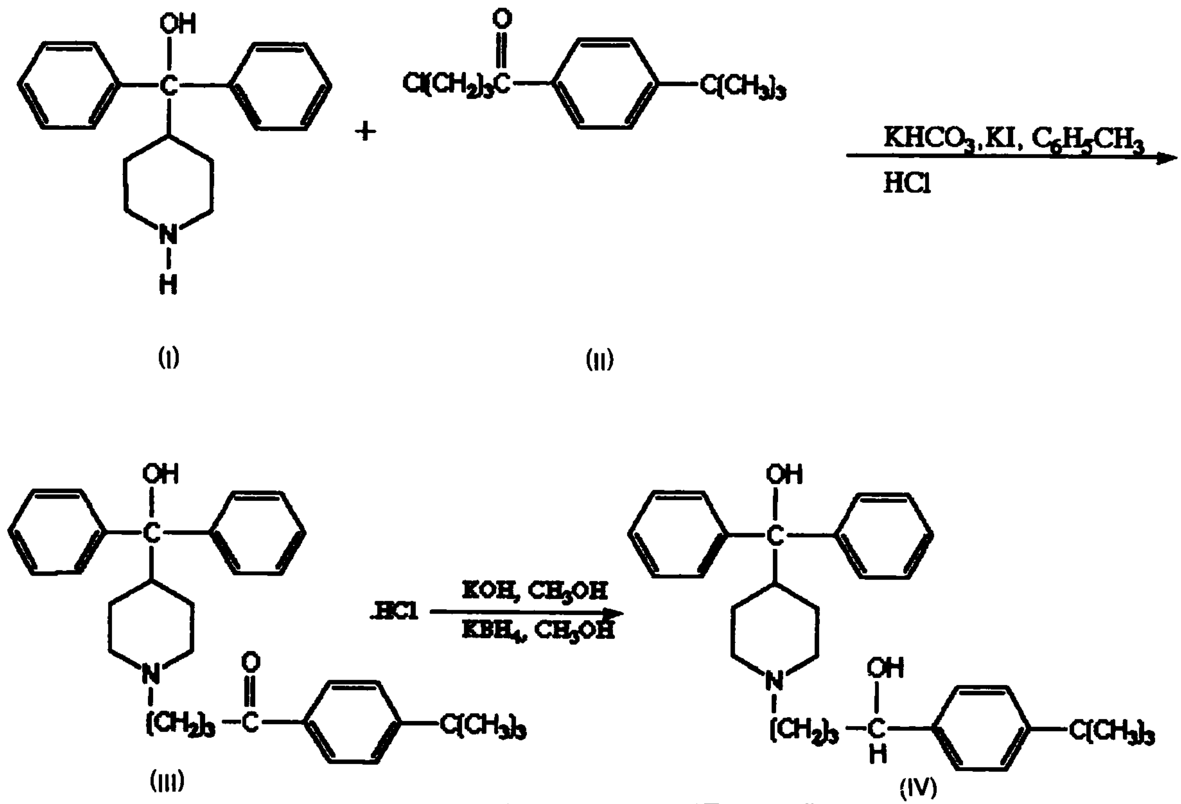


1.3. Colour, Appearance and Odour.

White, crystalline powder, odourless with very bitter taste.

2. SYNTHESIS

A mixture of ∝, ∝-diphenyl-4-piperidine methanol (I), 1- 4-(1,1-dimethyl ethyl) phenyl - 4-chloro- 1-butanone (II), potassium bicarbonate and potassium iodide in toluene was refluxed with stirring at room temperature. The warm reaction mixture was filtered and the cooled filtrate treated with excess ethereal hydrogen chloride. The resulting precipitate was recrystallized twice from a mixture of methanol/ isopropanol to give 1- 4-(1, 1-dimethyl ethyl) phenyl -4-4-(hydroxydiphenyl-methyl) -1-piperidinyl- 1-butanone hydrochloride (III). A solution of (III) in methanol was treated with a solution of potassium hydroxide in methanol until it was basic. The resulting mixture was cooled, stirred and treated portionwise with potassium borohydride. The cooling bath was removed and the reaction was stirred and then concentrated on a steam bath at reduced pressure to give a solid residue. After washing with water and two recrystallizations from acetone, terfenadine (IV) was obtained (1).



Scheme (1) Synthetic Pathway of Terfenadine.

3. POLYMORPHISM AND CRYSTAL HABITS

It appears that terfenadine could exist in various polymorphic forms. At present three distinct polymorphic forms and two solvates were identified. These were obtained from recrystallization of terfenadine from different solvents. Polymorph I: obtained by recrystallization from ethanol-water mixture. Polymorph II was obtained by recrystallization from methanol. Polymorph III (metastable) was obtained by recrystallization from propylene glycol.The differences in their physico-chemical properties are mentioned in the relevent subsequent sections. In commercial materials polymorph I and II coexist in different proportions. Polymorphic forms II and III could be converted to polymorph I by boiling in water. The presence of crystallization inhibitors retarded such transformation. In addition, terfenadine glass form could be obtained by rapid cooling of the drug melt. Crystals obtained from commercial material were euhedral having bladed and circular shape crystals, while polymorph III has a thin flake type powder. In this monograph the measurements were carried out mainly on the most stable polymorph I as described in the following sections (2).

4. PHYSICO-CHEMICAL PROPERTIES

4.1. Melting Range

USP specifies that the melting range of terfenadine is between 145-151°C (3). However, terfenadine exhibits different polymorphic forms having the following melting ranges (2).

Polymorph I (most stable)	149-152°C.
Polymorph II	146-148°C.
Polymorph III	142-144°C.

In addition, terfenadine melt forms glass upon cooling yielding glass transitions at 55.93°, 57.91°, 59.88°C(4).

4.2. Differential Scanning Calorimetry

Thermograms of the terfenadine stable, metastable polymorphic and glass forms are shown in figure (1a and b). These thermograms were obtained using Mettler TA3000 DSC-20 unit. The heating rate was 10°C min^{-1} and the sample size ranged between 3-10 mg. The drug has one endothermic peak, showing no signs of decomposition at its melting point. The following table illustrates the difference in heat of fusion and peak temperature of the known polymorphic forms.

	ΔH. J/G	Peak Temp.°C
Polymorph I	102.0	151.1
Polymorph II	100.5	147.8
Polymorph III	82.4	143.5

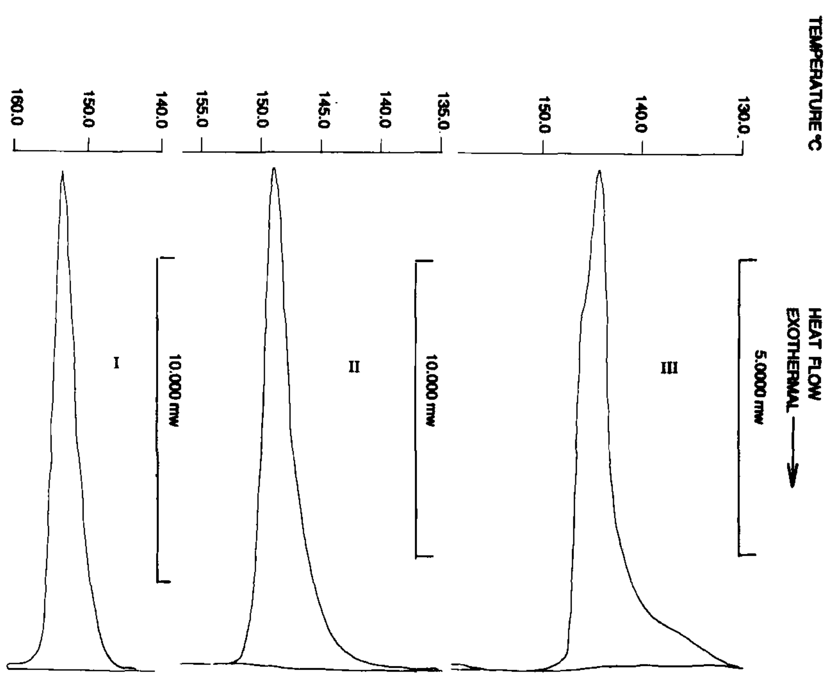


Figure (1a): DSC Curves of Terfenadine Polymorphic Forms I, II and III.

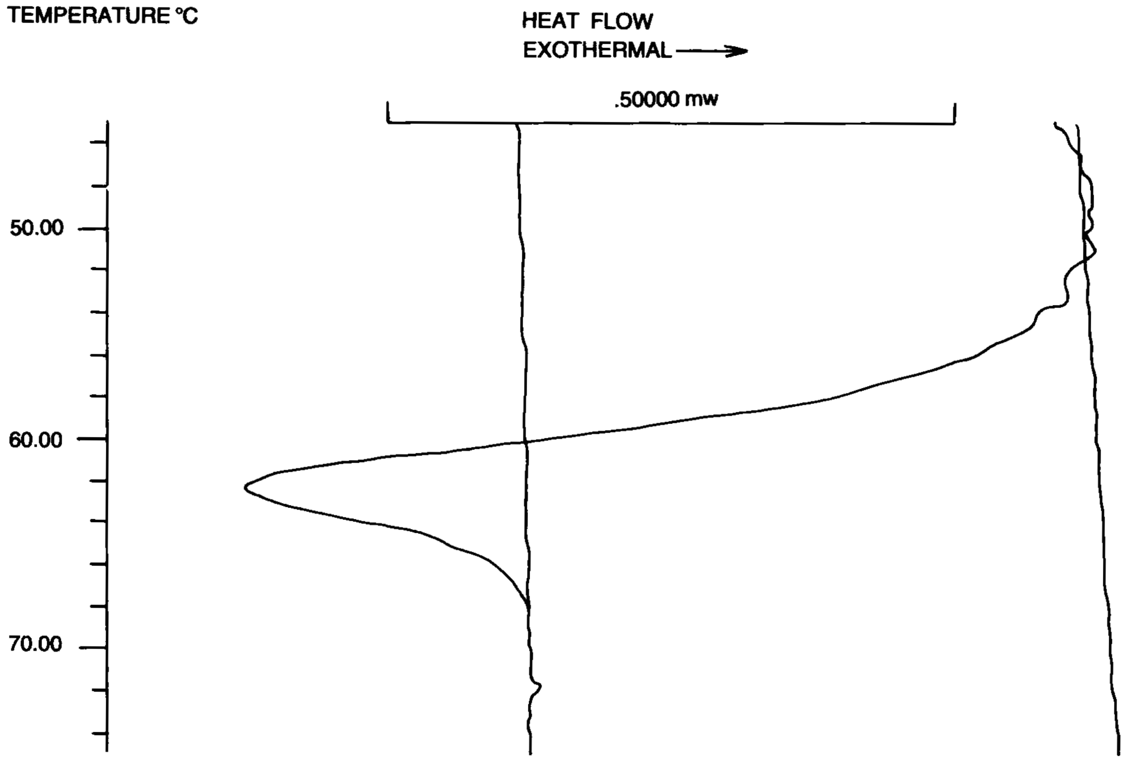


Figure (1b): DSC Curve of Terfenadine Glass Form.

4.3. Solubility

The solubility of the most stable polymorph of terfenadine was determined by shaking the excess of the drug in various solvents till equillibrium at 30°C. Equillibrium solubility is presented in table (I).

TABLE (I)

Terfenadine Equillibrium Solubility in Different Solvents at 30°C

Solvent	Solubility Gm/ 100ml 30°C
Water	0.001
Ethanol	3.780
Methanol	3.750
Hexane	0.034
0.1M Hydrochloric Acid	0.012
0.1M Citric Acid	0.110
0.1M Tartaric Acid	0.045

Terfenadine is highly hydrophobic, tends to adsorb on surfaces. It's solubility is slightly improved in mineral acids but increases appreciably in relatively high concentrations of hydroxy acids (5). It was shown that solubility improved by the addition of even low concentrations of sodium chloride to hydrochloric acid-terfenadine solutions and monobasic sodium phosphate to phosphoric acid-terfenadine solution (6). The drug solution in ethanol and methanol is sticky and deposits terfenadine glass form when the solvent evaporates (5).

Terfenadine citrate and tartarate has a distinct surface activity and their solubility is pronouncely higher than terfenadine. Different polymorphic and glass forms showed gradual transformation to the stable form in the presence of water. The dissolution rate was highest for the glass form followed by polymorph III and polymorph II, respectively. In addition, solubility increased appreciably by complexing with cyclodextrins and polyethylene glycols.

4.4. Dissociation Constant

The pKa of terfenadine is 10. This is an approximate value as reported earlier(7). However, pKa of terfenadine hydrochloride was determined by titration against 0.01M potassium hydroxide, both dissolved in different concentrations of methanol-water mixtures. The apparent pKa was obtained by extrapolation technique(8) and was 6.56 at 22°C(9).

4.5. Spectral Properties

4.5.1. Ultraviolet spectrum:

Terfenadine solution in methanol was screened between 220-350nm using Beckman DU7 spectrophotometer. It exhibited two maxima at 260nm and 225nm with a shoulder at 254nm, figure (2).

The molar absorptivities for terfenadine in some selected commonly used solvents are shown in table (II).

TABLE (II)

Molar Absorptivities of Terfenadine in Some Commonly Used Solvents

Solvent	Max.Wavelength nm	Molar Absorptivities
Methanol	260	660.4
Ethanol	260	671.7
Dichloromethane	260	762.2

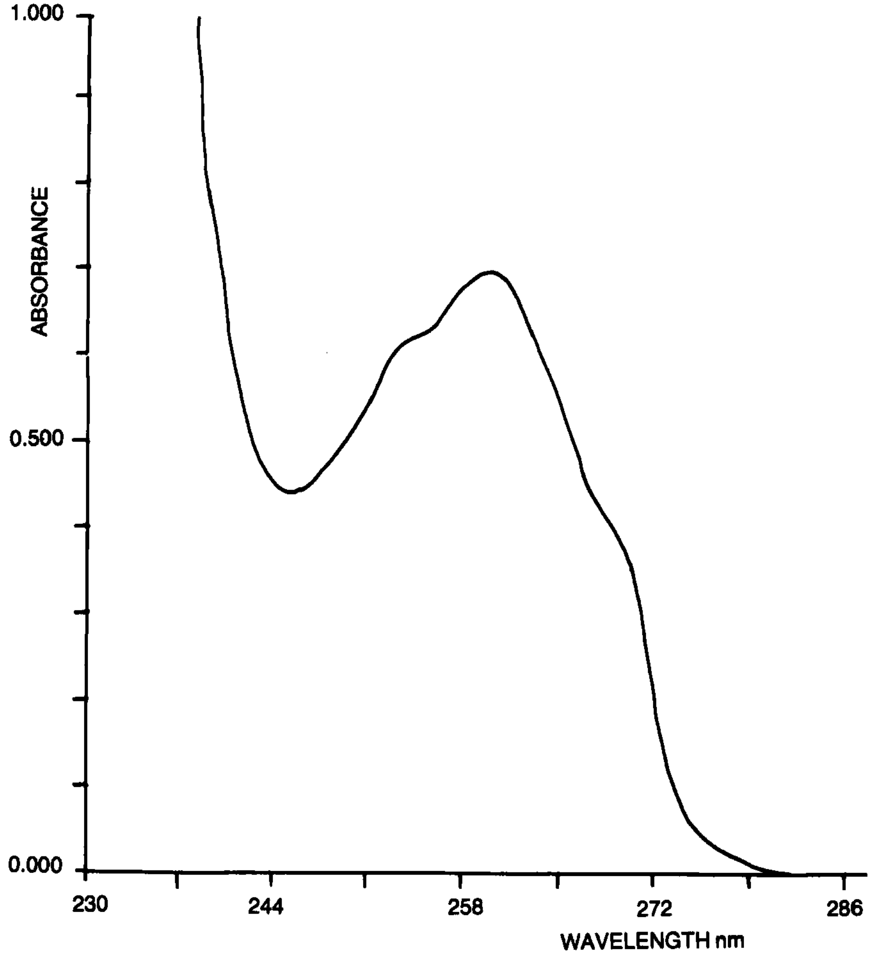


Figure (2): The UV Absorption Spectrum of Terfenadine in Methanol.

4.5.2. Flourescence spectrum

Terfenadine solution in buffer pH2 (citric acid-sodium dihydrogen phosphate) showed excitation and emission maxima at 260 and 289nm respectively. The spectrum was recorded on Kontron SFM25 spectrofluorometer, figure (3).

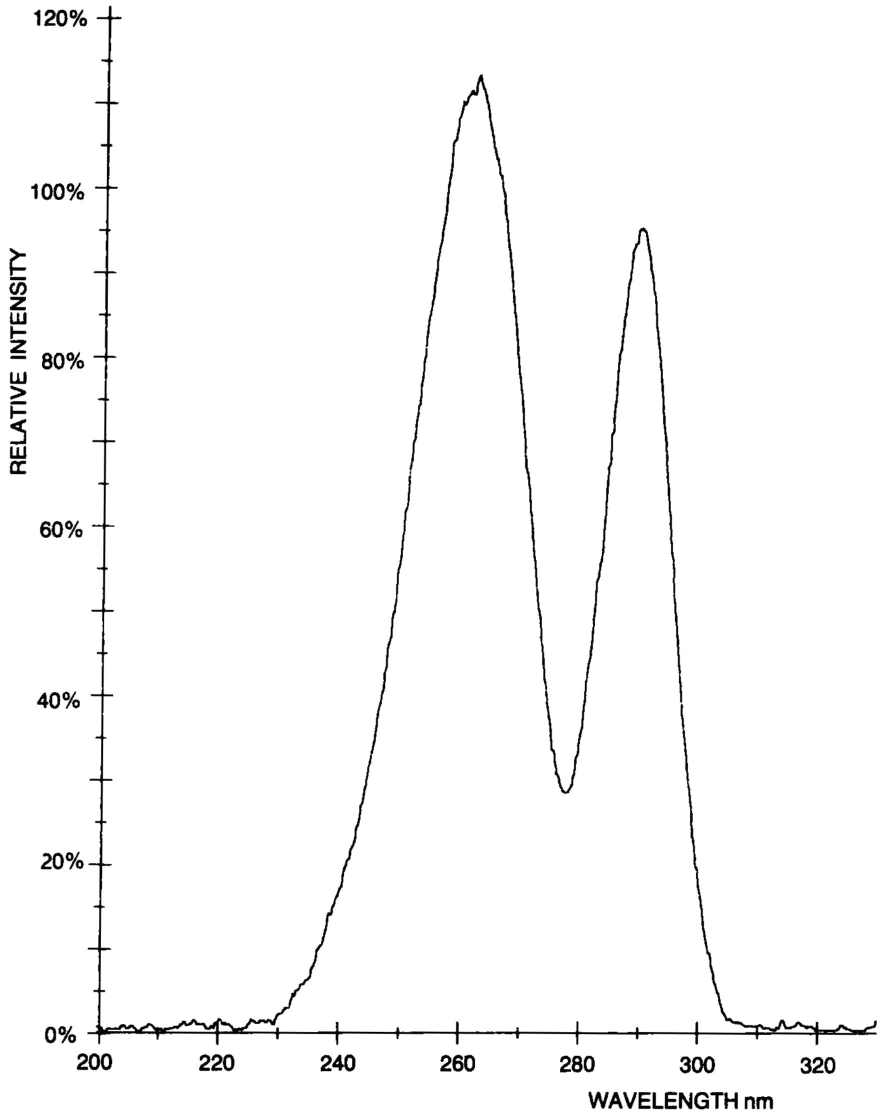


Figure (3): The Fluorescence Excitation and Emission Spectrum of Terfenadine in Buffer solution pH 2.0.

4.5.3. Infrared spectrum

Terfenadine infrared spectra presenting polymorph I,III and the glass form prepared as a dispersion in KBr were obtained using Shimadzu IR-435 spectrophotometer, figure (4a,b and c). Spectral assignments for principal absorption bands of polymorph I are given in table (III) showing consistency with the proposed structure. I.R. spectra of polymorphic forms I and III showed little differences. Nevertheless some differences were observed in the regions 1400-1300 cm^{-1}, 1300-1150 cm^{-1}, these regions are responsible for C-H bending and C-O streching in these polymorphic forms.

However, glass terfenadine showed a distinct difference in the region 3500-3200 cm^{-1} where polymorph III displayed a band at 3500 cm^{-1} and a shoulder at 3400 cm^{-1}. Glass form showed broad bands at 3400 cm^{-1} and 3300 cm^{-1}. Such broadness may be due to intermolecular hydrogen bonding of OH group. Generally, the differences in terfenadine polymorphic forms are very negligible indicating the lack of intramolecular interaction.

TABLE (III)

Band Assignments of Infrared Spectrum of Terfenadine

Band Frequency cm^{-1}	Functional Group
3250, 3240	O-H stretching vibration for two hydroxyl groups.
3000	C-H stretching vibration for aromatic hydrogens.
2900	C-H stretching vibration for the aliphatic hydrogens.
1370, 1390	C-H streching for tert-butyl group, doublet.
1150, 1100	C-O streching vibration for C-OH groups of 3° and 2° alcohols, respectively.
830	C-H out of plane bending for aromatic C-H of para disubstituted benzene.
750, 630	C-H out of plane bending for aromatic C-H of mono substituted benzene.

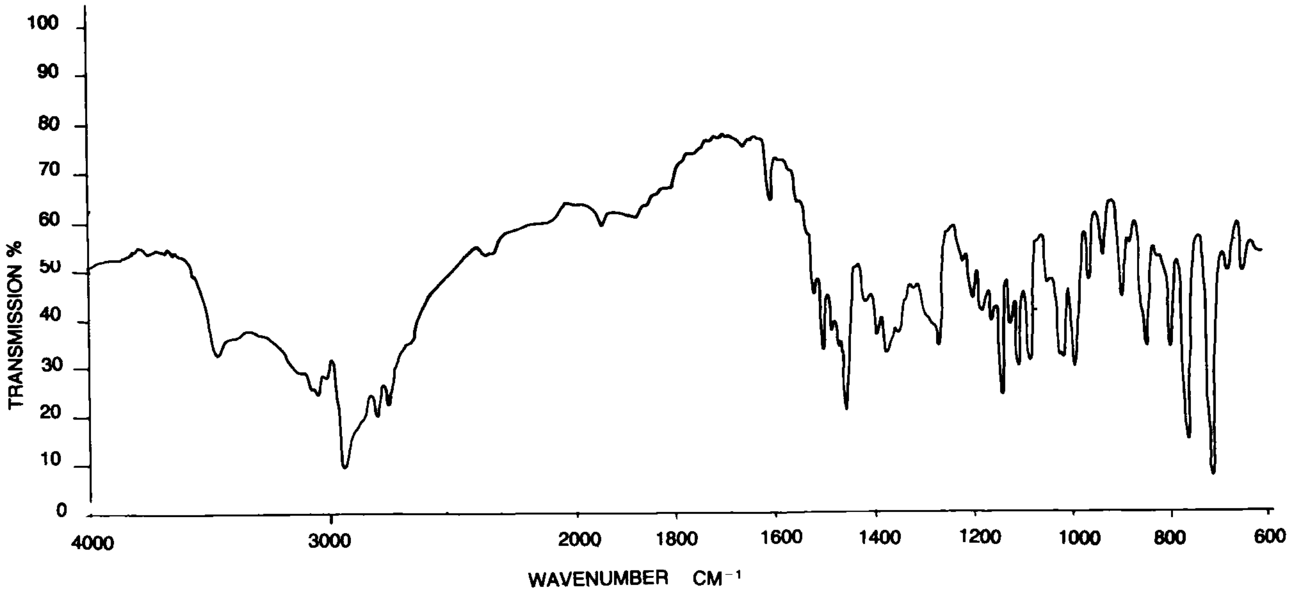


Figure (4a): I.R. Spectrum of Polymorph I. - KBr Disc.

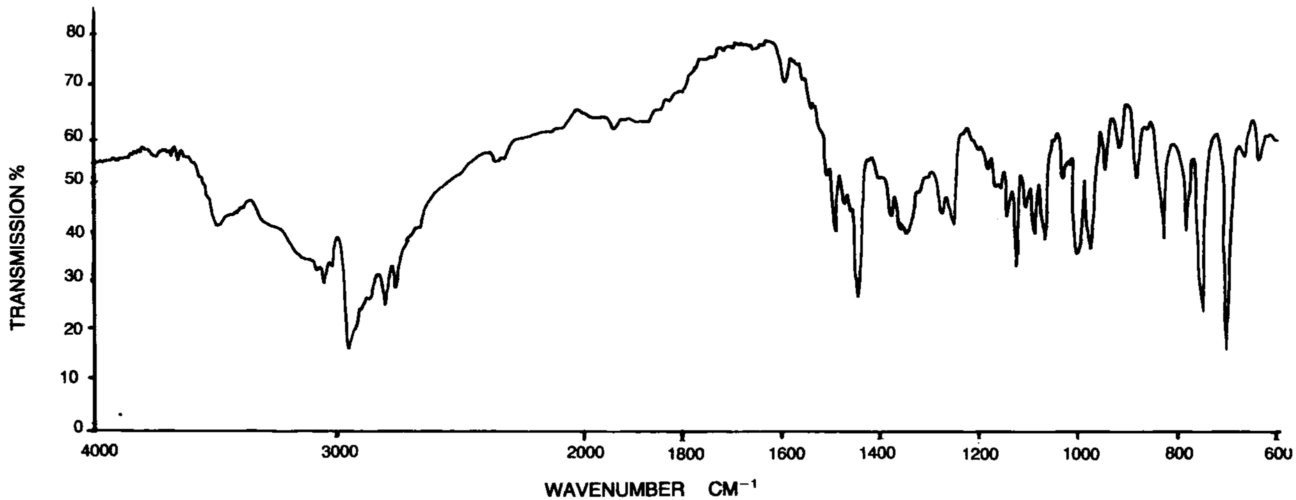


Figure (4b): I.R. Spectrum of Polymorph III - KBr Disc.

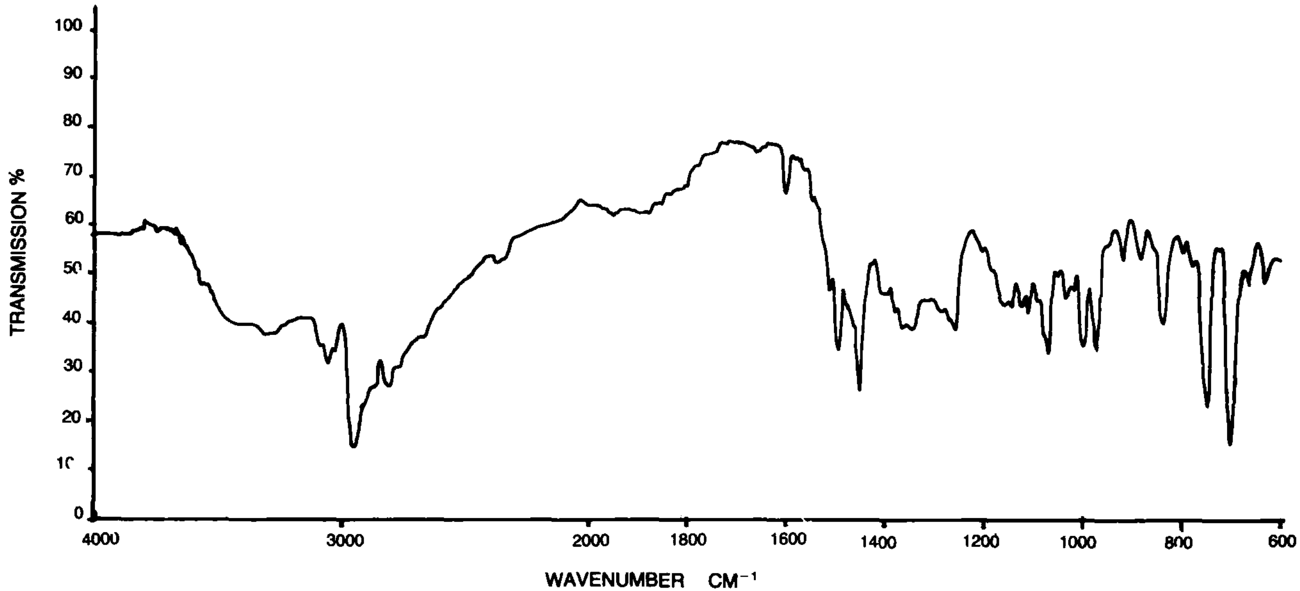


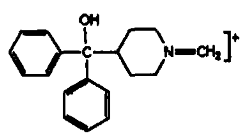
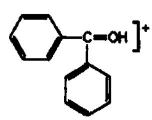
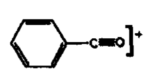
Figure (4c): I.R. Spectrum of Glass - KBr Disc.

4.5.4. Mass spectra

4.5.4.1. Electron impact (EI): The electron impact mass spectrum of terfenadine is shown in figure (5). The spectrum was obtained by direct solid insertion probe at electron energy of 70 eV using Hitachi Perkin Elmer RMU-6H mass spectrometer. The spectrum shows a molecular ion peak M^+ at a mass/charge (m/z) ratio of 471 and a base peak at 280 corresponding to the α-cleavage at the nitrogen atom. Other fragments including diphenylhydroxymethyl ion and tert-butyl radical were displayed as well (at m/z = 183, 57). The pertinent fragments, their relative intensities and proposed structure are presented in table (IV). The mass spectra of the dehydration product of terfenadine was previously reported showing ion peak at m/z 453 (M^+, 280 and 183) (10).

TABLE (IV)

Mass Spectral (Electron Impact) Assignment for Terfenadine

M/z	Relative Intensity%	Fragment Ion
471	55.6	$M]^+$
453	4.3	$M - 18]^+$
280	100	
183	13	
105	13	
57	18	$C_4H_9]^{\bullet}$

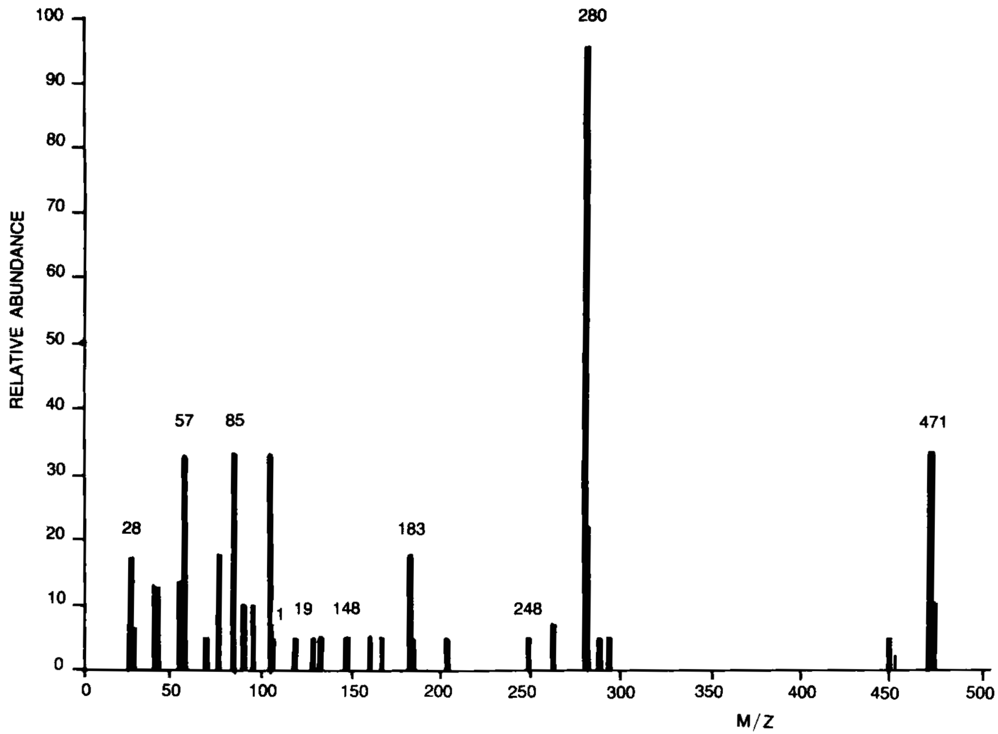


Figure (5): The Electron Impact Spectrum of Terfenadine.

4.5.4.2. Chemical ionization (CI): The spectrum was recorded on Varian MAT-44 spectrometer, figure (6). The spectrum shows a prominant pseudo molecular ion at m/z ratio of 472 and an ion peak at 454 corresponding to the dehydrated fragment.

4.5.5. Nuclear magnetic resonance

4.5.5.1. Proton magnetic resonance: The NMR spectrum was obtained on a Bruker WP 80 SY instrument of a solution of terfenadine in deuterated chloroform. The spectrum is presented in figure (7). The assignment of the protons and their multiplicity pattern is listed in table (V). Difficulty was encountered in assigning the protons of the hydroxyl groups. One of the protons was masked by the multiplet corresponding to the aliphatic protons absorbing at 2.0-2.4 ppm. This assumption was supported by a deuterium exchange experiment. However, the exchange did not reveal the possible absorption assignment for the second hydroxyl proton. Using dimethylsulfoxide as a solvent alone or with D_2O, was not indicative due to the change in the absorption spectrum which could be explained by the hydrogen bonding properties of the solvent. Therefore, it could be assumed that the second hydroxyl proton absorbs in the same range as the multiplets of aliphatic protons extending from 1.9-2.4 ppm.

TABLE (V)

Proton NMR Assignments of Terfenadine

Chemcial Shift H ppm	**Relative Number of protons (pattern)**	**Assignment**
7.4 - 7.5	14 Two doublets	Disubstituted aromatic rings protons.
7.19 - 7.26	Singlet	Monosubstituted aromatic rings protons
4.5 - 4.59	1 (t, broad)	C_1H
2.8 - 3.1	2 (m, broad)	C_4H_2
2.0 - 2.4	5 (m, broad)	$C_2'H_2$, $C_6'H_2$, O-H
1.5 - 1.9	10 (m, broad)	C_2H_2, C_3H_2, piperidine ring protons at C_3', C_4', C_5', O-H
1.29	9 (s, sharp)	Tert-butyl protons

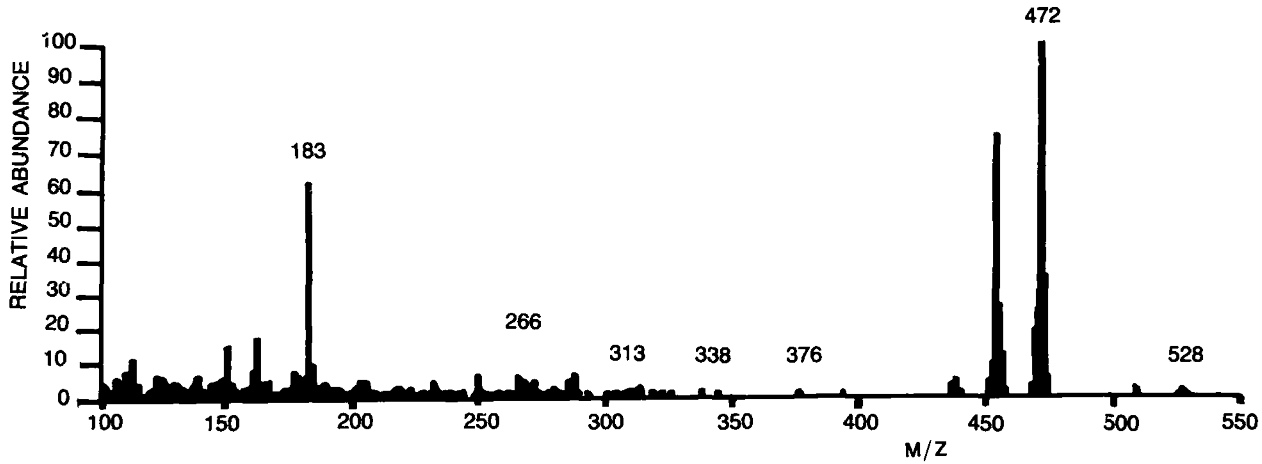


Figure (6): The Chemical Ionization Spectrum of Terfenadine.

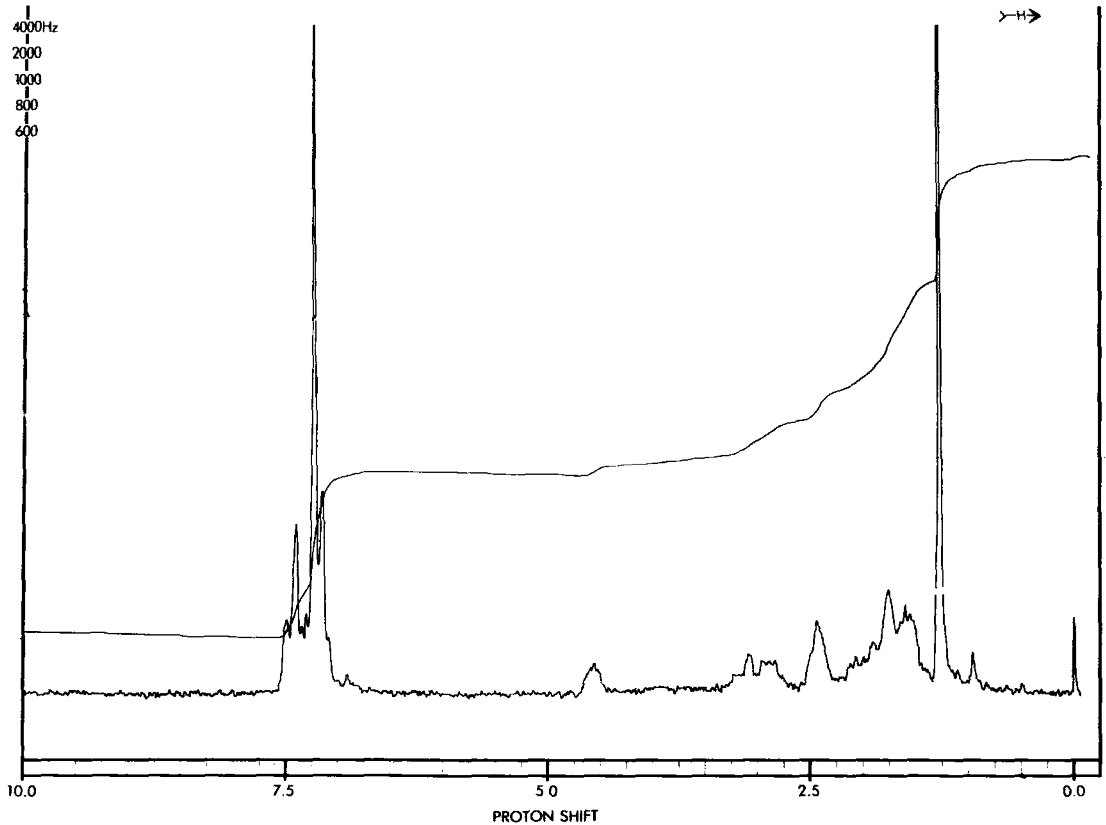


Figure (7): Proton NMR Spectrum of Terfenadine.

4.5.5.2. Carbon - 13: Carbon-13 NMR spectra were recorded on Varian FT-80A spectrometer. The assignment, listed in table (VI) was further confirmed by carrying out an inverse polarization transfer study. The recorded spectra are shown in figure (8).

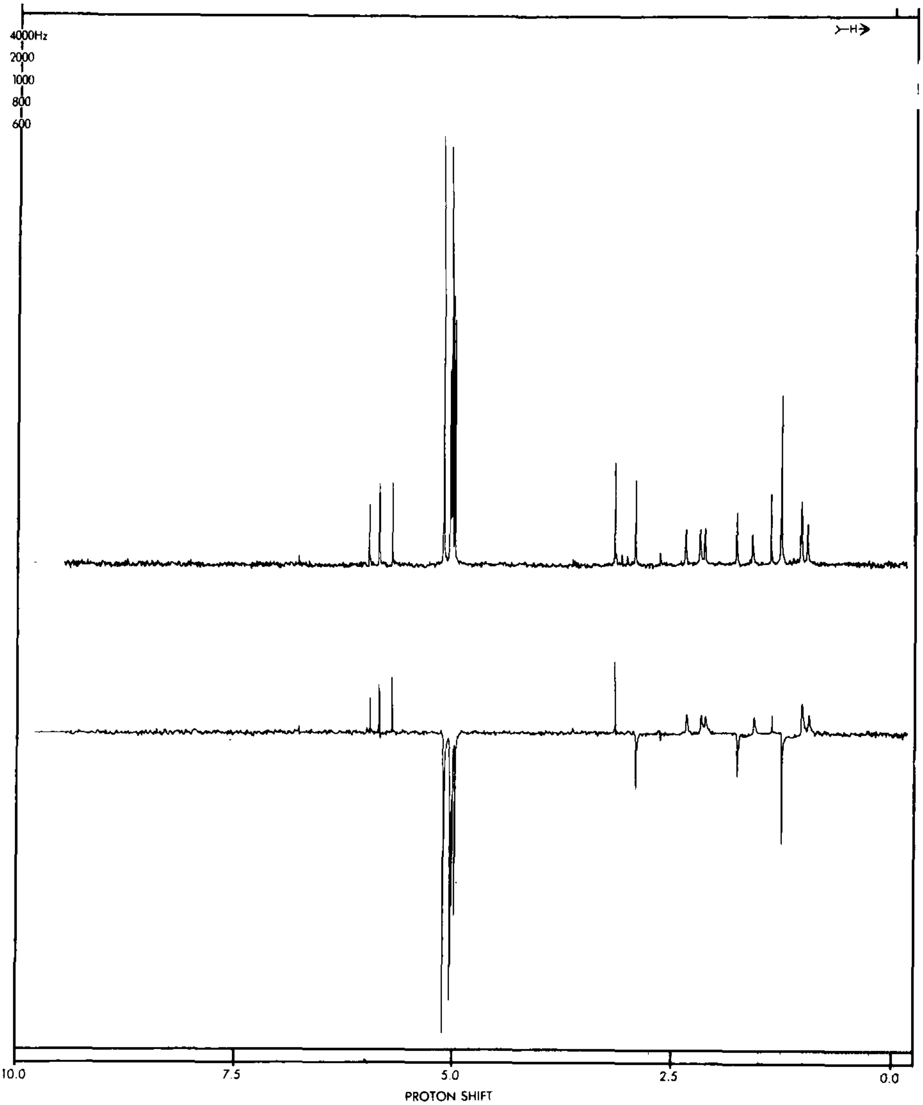


Figure (8): [13]Carbon NMR Spectrum of Terfenadine in DMSO.

TABLE (VI)

Carbon - 13 NMR Assignments of Terfenadine

Chemical Shift C ppm	Assignment
24.17	C_3
25.93	C_3', C_5'
31.60	tert-butyl C ($\underline{C}H_3$)
34.50	tert-butyl $\underline{C}$. (CH_3)
39.75	C_2
44.26	C_4'
53.45	C_2'
54.80	C_6'
58.90	C_4
73.41	C_1
79.33	C_α
125.01	C_2'', C_6''
125.59	C_3'', C_5''
125.96	ortho carbons-benzhydryl
126.35	para carbons-benzhydryl
128.16	meta carbons-benzhydryl
142.81	C_4''
146.49, 146.62	Epsi carbons-benzhydryl
149.39	C_1''

4.5.6. X-Ray powder diffraction

The x-ray powder diffractions of I and III polymorphic forms of terfenadine were determined by Philips PW 1050-81 Goniometer with a PW 1729 Generator, It was equipped with nickle filtered copper radiation with λ = 1.541 nm. The interplanner distance and relative intensity of the major peaks for the stable and metastable forms of terfenadine are listed in tables (VII and VIII). Figure (9a,b) presents x-ray powder diffraction pattern for polymorph I and III.

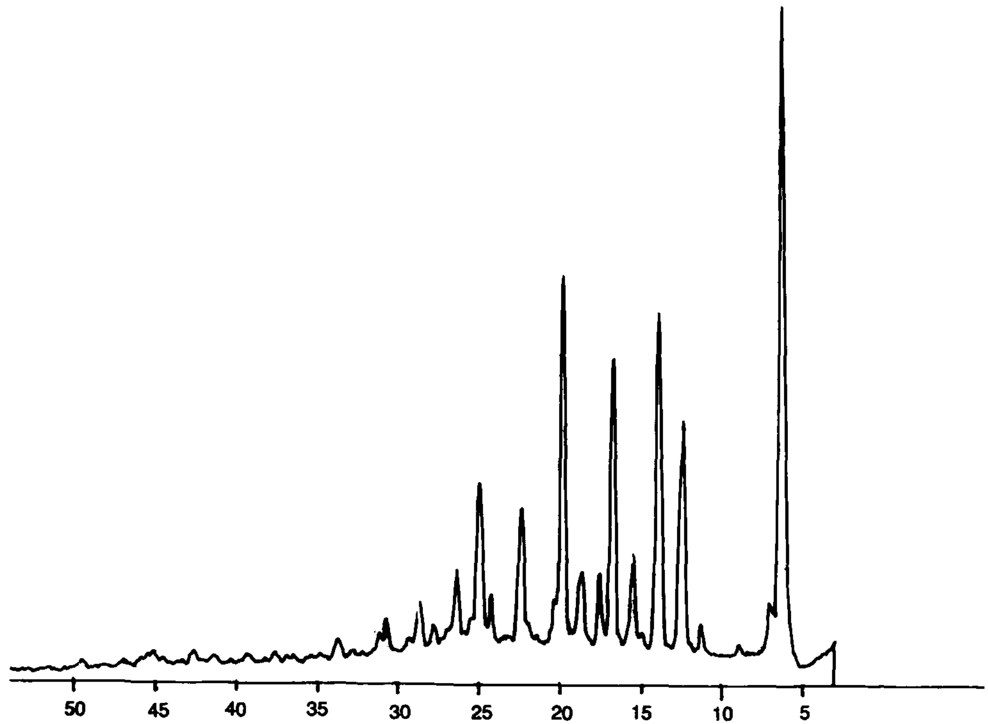


Figure (9a): Powder X-Ray Diffraction Pattern of Terfenadine Polymorph I.

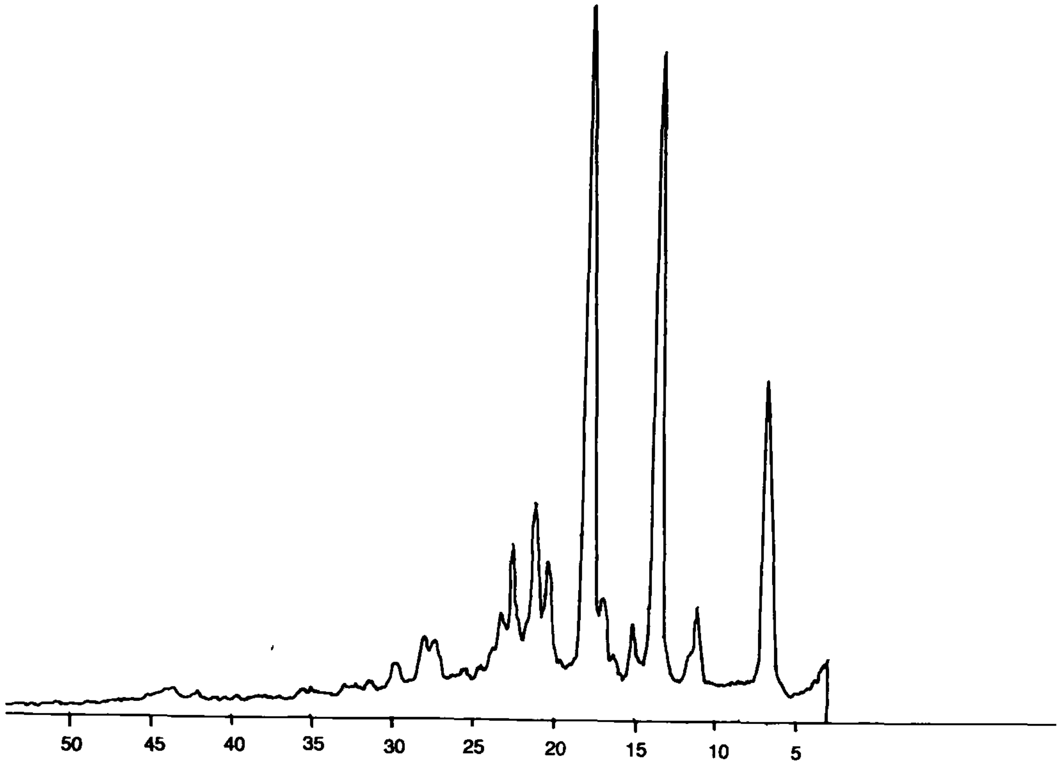


Figure (9b): Powder X-Ray Diffraction Pattern of Terfenadine Polymorph III.

TABLE (VII)

X-Ray Powder Diffraction of Terfenadine Polymorph I

Peak Angle (2θ)	Interplanner Distance d* (°A)	**I/I max (%)
06.4	13.81	100
07.3	12.11	04.32
12.5	07.08	35.8
12.1	06.28	51.2
15.6	05.68	13.82
16.6	05.34	43.2
17.5	05.06	09.87
18.5	04.79	09.87
19.8	04.48	55.5
22.3	03.98	20.9
24.3	03.66	05.55
25.0	03.56	22.2
26.3	03.38	10.49
27.9	03.19	02.83
28.5	03.13	06.17
30.5	02.93	03.7

• $d = n/2 \sin \theta$

** Rased on the highest intensity of 1.000

TABLE (VIII)

X-Ray Powder Diffraction of Terfenadine Polymorph III

Peak Angle (2θ)	Interplanner Distance d* (A°)	**I/I max (%)
07.0	12.62	46.38
11.3	07.83	10.24
13.6	06.51	93.37
15.3	05.79	06.62
17.2	05.15	05.42
18.0	04.93	100
20.3	04.37	12.04
21.4	04.15	18.67
22.7	03.92	14.45
23.5	03.78	04.21
27.4	03.25	03.61
28.0	03.18	03.61
29.8	02.99	03.01

• $d = n/2 \sin \theta$.

** Based on the highest intensity of 1.000

5. METHODS OF ANALYSIS

5.1. Starting Material and Pharmaceutical Dosage Forms

5.1.1. Elemental analysis

	Calculated for $C_{32}H_{41}NO_2$	Found
C	81.45	81.48
H	8.76	8.77
N	2.97	2.96

5.1.2. Non-aqueous titration

Terfenadine was dissolved in glacial acetic acid and titrated against 0.1 M perchloric acid. Each ml of 0.1M perchloric acid is equivalent to 47.169 mg of the drug. As anticipated this method suffers from excipients interferences hampering its use in dosage forms (3 and 11).

5.1.3. Spectrophotometric methods

5.1.3.1. Ultraviolet absorption: Terfenadine was dissolved in a mixture of methanol, acetic acid and water in the following proportions (50:6:44), respectively. The absorbance was read at two maxima 260nm and 238 nm. This method could be applied to concentrations ranging, 0.1-0.6 mg.mL^{-1}. However, the extension coefficient is low and the interference from additives is clearly observed(11).

5.1.3.2. First derivative: A method for terfenadine determination, by first derivative peak height amplitude measurements, at two different wavelengths is possible, (Figure 10). The accuracy and precision of the method is demonstrated with 99.3% recovery and less than 1% standard deviation for solutions concentration ranging 0.1-1.0 mg. mL^{-1} in methanol at 271.5 nm. Beer's Lamberts law was obeyed for dissolution testing of lower concentrations ranging 0.005 mg to 0.006 mg. mL^{-1} at 224.2nm in 0.1M HCl. The described method have shown to eliminate the background distortion due to excipients. This method could be applied to tablets, suspension and capsules (12).

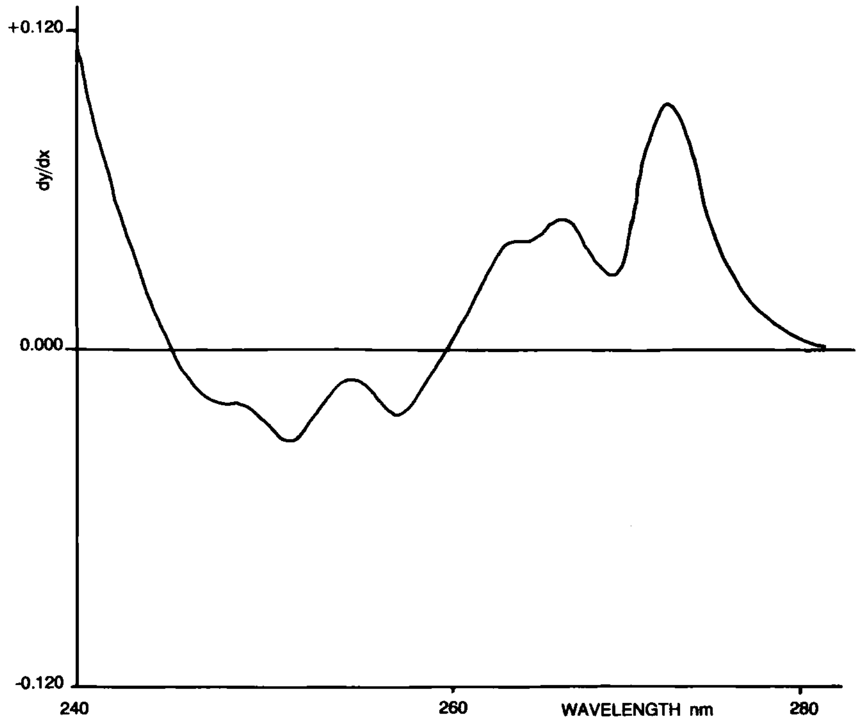


Figure (10): The First Derivative Spectrum of Terfenadine in Methanol.

5.1.3.3. Colourimetric spectroscopy:

a - Terfenadine formed an ion pair complex with methyl orange which was extracted in dichloromethane and showed maxima at 427 nm. The colour obtained was stable and the complex obeyed Beer's Lambert law between 0.004-0.016 mg.mL^{-1}. The optimum pH of the aqueous layer was ranging from 3.5-6.0. This method was applied for tablets, capsules, suspension and content uniformity testing(11).

b - A bluish green colour resulted from the reaction between terfenadine and 7,7, 8,8-tetracyanoquinodimethane. The absorption was measured at 845 nm. Beer's law is obeyed over the terfenadine concentration range 0.002-0.012 mg. mL^{-1}. This method is applicable to tablets(13).

c - Terfenadine iodine charge transfere complex was formed and the absorption was measured at 290nm. This method could be applied to tablets having concentrations 2-12μg.mL^{-1}(13).

5.1.4. Chromatographic methods

5.1.4.1. Thin layer chromatography: Thin layer chromatography system (TLC) was developed to assay terfenadine in the bulk material. The mobile phase consisted of methanol, butanol, water, toluene and acetic acid mixed in proportions of 2.0:3.0: 1.0:2.0: 0.1, respectively. The solution of terfenadine in methanol (2-10mg.mL^{-1}) was spotted with 5μL micropipette on precoated silica gel plates (10 × 10 cm). The developed plates were read at 254 nm, a linear response for concentrations ranging from 2-10mg.mL^{-1} was obtained(11).

5.1.4.2. High pressure liquid chromatography:

a - Synthesis Impurities: The method for synthetic impurities is an official test described in the current USP (3).

b - Bulk and Dosage Forms: Various HPLC systems were reported for terfenadine analysis in bulk and dosage forms. A brief description for these applied systems is given:

1 - Reverse phase column (C_{18}) with a mobile phase consisting of a mixture of 0.1 triethylammonium acetate buffer having pH5, acetonitrile and methanol at ratios of 6.25: 6.25: 87.5

respectively. The injection loop used was 10 μL. The internal standard was ephedrine hydrochloride with retention time 3.4 min., flow rate was 2 mL.min^{-1}. The terfenadine retention time was 4.2 min. and detection was carried out at 254 nm and could be obtained in a limit of 0.1-0.8 mg.mL^{-1}(14).

2 - Ion pair HPLC assay using ODS column (5 μm) with a mobile phase consisting of 0.01M n-hexane sulphonate in acetonitrile (80 parts) water (20 parts) containing 0.1% glacial acetic acid. The maximum limit of injection was 170 μL and the flow rate was 1 mL.min^{-1} with quinine as internal standard. The retention times of quinine and terfenadine were at 4.7 and 6.7 min, respectively. The detection was carried out at 218 nm (0.64 AUFS). Terfenadine-quinine peak area ratio was linear over 68-544 ng of terfenadine injected(15).

3 - Reverse phase column spherisorb ODS-2 (3-μm) was used with a mobile phase of 60/40 v/v acetonitrile/ water made with 0.012M in sodium phosphate buffer (ca pH 2.3) and 0.021M in sodium perchlorate. Injection volume of 20 μL was used. The flow rate was 1.5 mL.min^{-1}. The detection was carried out at 210 nm at 0.23 AUFS. Retention time for terfenadine was 4.5 min. This stability indicating method was used to assay the drug (0.3-3.0mg.mL^{-1}) in the presence of ibuprofen and pseudoephedrine hydrochloride in aqueous solutions containing tween 80 and methyl cellulose(16).

4 - Reverse phase column (C_{18}) Bonda Pack with a mobile phase consisting of 0.25M sodium acetate buffer (pH5) added to acetonitrile (1:1 v/v). The injection loop was 20 μL and the flow rate was 1 mL.min^{-1}. the detection was carried out at 225 nm. The internal standard was thiothxane having retention time 9.8 min., while the retention time of terfenadine was 14.5 min. This method achieved measurement between 10-80 μg.mL^{-1}(17).

5 - Zorbax C-8 column, (6 μm), (15 cm × 4.6 mm as internal diameter) was used. Mobile phase consisted of acetonitrile-0.1M triethylammonium phosphate buffer (pH7) mixed in 70:30 v/v ratios. The loop used was 100 μL. The flow rate was 1.5mL.min^{-1} and the measurement was monitored at 260 nm and by flouresence detector (EX260 nm and EM289 nm). The retention time was 4.8 min. This method could differentiate various degraded products from the parent compound. The method showed linear UV (260nm) response between 6 and 60 μg injected (10).

5.2. Body Tissues and Fluids.

5.2.1. RadioImmunoassay:

The method consisted of extracting terfenadine from alkalinized plasma into hexane. Aliquots of the hexane extract were evaporated to dryness. To this residue was added tritium-labelled terfenadine in aqueous ethanol, phosphate buffered saline and terfenadine antisera. The mixture was then incubated overnight at 4°C and unbound terfenadine subsequently removed with charcoal. After centrifugation, the supernatant was decanted into scintillation vials, mixed with scintillator (0.6% Omniflour, New England Nuclear, in a 2:1 mixture of Toluene/ Triton X-100) and counted in a Beckman LS-300 Liquid Scintillation Spectrometer. The counts per minute (cpm) of the unknown plasma samples were compared to a standard curve that was processed along with the sample. The recovery of terfenadine through the extraction procedure was shown to be 90%(18).

5.2.2. ^{14}C analysis

Analysis of ^{14}C concentrations in all biological samples from the material balance study was by combustion (faeces were homogenized with 9 volumes of water) of dried residues in a Packard Model 306B sample Oxidizer followed by liquid scintillation counting in a Packard Tri Carb Scintillation Counter (Model 3320). Quench correction was by automatic external standard channel ratio method(18).

5.2.3. Gas chromatography-Mass spectrometry

A Dupont 321 GC/MS was used to analyse terfenadine metabolites. Two types of urine extracts were subjected to GC/MS analysis for structure elucidation, namely a pH12 toluene extract (metabolite II) (18), and pH4 ethyl acetate extract (metabolite I). The GC/MS conditions were as follows for:

Metabolite II: 6 ft glass column, packed with 5% OV-17, (Chrom WHP, 100/120 mesh); injector, column and separator temperature: 250°C; ionization voltage: 70 eV; mass range: 60-400 (amu); integration time: 10 ms/amu.

Metabolite I: 2 ft glass column packed with 1% Dexsil (Chrom WHP, 100/120 mesh); injector temperature: 250°C; column temperature: 280°C; separator temperature: 250°C; ionization voltage: 70 eV; mass range: 250-750 (amu); integration time: 10 ms/amu.

6. STABILITY

6.1. Stability of the Solid

Terfenadine powder is stable against heat and light. No degradation products where observed after 12 months storage in amber colour glass at 40°C and under room light conditions. The solid dosage form is still stable for 4 years during storage at room temperature (10, 19).

6.2. Stability in Solution

Terfenadine dispersion has excellent stability at pH 5-11 when protected from intense light. A low level of degradation is observed at pH 1.5 in darkness after 25 weeks storage. The stability of terfenadine is not effected by oxygen if the solution is protected from light. Even under intense flourescent light, terfenadine remains stable for up to 8 weeks storage at 27°C(10).

7. PHARMACOKINETICS

Australian National Drug Information Service compiled a short but thorough profile on terfenadine pharmacology and therapeutics. The following part concerning pharmacokinetics was extracted as produced from that profile which has a useful reference list(7).

7.1. Absorption

Terfenadine is completely and rapidly absorbed from the gastrointestinal tract and undergoes extensive biotransformation (over 99%) probably by first-pass metabolism in the liver. Mean peak plasma concentration after administration of a 60 mg tablet was 0.84 ± 0.43 (range 0.26-1.92) nanograms.mL^{-1}, (measured by radioimmunoassay (RIA)) which was reached in 1.74 ± 1.47 (range 0.5-12) hours. A linear correlation was noted between dosage and peak plasma concentration after single doses of 60 and 180 mg terfenadine suspension. Mean peak plasma concentrations for these doses were 1.544 ± 0.726 and 4.519 ± 2.002 nanograms.mL^{-1}, respectively, and

were achieved in approximately 1 hour. However, Area Under the Curve (AUC) calculations indicated non linear kinetics with an almost four-fold increase in peak plasma concentration for the three-fold increase in dose.

7.2. Bioavailability

Tablet and suspension formulations were found to be bioequivalent. The AUC for 0-48 hours values were not significantly different. For example AUC for 60 mg suspension was 11.4 hour nanograms.mL^{-1} and for 60 mg tablet 10.3 hours nanograms.mL^{-1}. The 180 mg tablet AUC was 39.2 hours nanograms.mL^{-1} while the 180 mg suspension AUC was 43.2 hours nanograms.mL^{-1}. Peak plasma levels (PPL), were higher for the suspension than for the corresponding tablet doses, for 60 mg dose the PPL for tablets and suspension were 0.84 and 1.44 nanograms.mL^{-1}, respectively. When 180 mg doses were given the PPL for tablets and suspension were 2.58 and 4.46 nanograms.mL^{-1}, respectively. These data indicate that the drug is more rapidly absorbed from the suspension but the total quantity of drug absorbed from the two formulations is similar.

7.3. Distribution

Animal studies indicate that labelled terfenadine is distributed widely with highest concentrations occurring in liver and gastrointestinal tract supporting the assertion that biliary mechanisms play a major role in the metabolic disposition of terfenadine. Levels of terfenadine in rat brain were low after IV dosing of 10mg/ kg and not detectable after a similar oral dose. There is no information available on the distribution of terfenadine in humans.

7.4. Protein Binding

Terfenadine is 96 to 97% bound to human serum albumin.

7.5. Metabolism

Comparisons of plasma concentration data derived from RIA and C-14 labelled studies indicate that the degree of terfenadine biotransformation in humans is over 99% and that first pass metabolism may play a major role in the disposition of terfenadine. Three metabolites have been identified in man: the carboxylic acid analogue of terfenadine (metabolite I), and its corresponding ester and the piperidine carbinol derivative (metabolite II). The latter metabolite is a synthetic precursor of terfenadine and has no significant antihistamine activity. The other metabolites do display antihistamine activity in the isolated guinea pig ileum. Although relative antihistamine potency was not investigated thoroughly, the carboxylic acid derivative (metabolite I) is probably about 30% as potent as terfenadine. Terfenadine undergoes oxidation of one of the methyl groups of the t-butyl substituent to produce metabolite I which is probably formed from the oxidation of an intermediate alcohol. This metabolite is found in both urine and faeces. Metabolite II is found primarily in urine and presumably arises from oxidative dealkylation of the substituted butanol side chain attached to the piperidine nitrogen atom.

7.6. Excretion

Terfenadine is excreted mainly in the faeces and to a lesser extent in the urine as metabolites. Approximately 40% of a radioactive labelled dose of terfenadine appeared in the urine in 5 days whilst 60% was recovered in the faeces in 12 days. Metabolites I and II accounted for 38 and 33% of the urinary radioactivity respectively, whereas (metabolite I) accounted for 49% of the faecal radioactivity.

7.7. Half-Life

The distribution half-life of terfenadine is 3.4 hours and the elimination half-life is 20.3 hours.

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CUMULATIVE INDEX

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